Cellular and Vaccination-based Immunotherapy of Acute Myeloid Leukaemia

Krishnamurthy, Pramila

Awarding institution:
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Pramila Krishnamurthy

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Declaration

I hereby declare that I alone composed this thesis and that the work is my own except where stated otherwise.

Pramila Krishnamurthy

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Finally, I dedicate this thesis to my husband, who met me before I began this project and has seen it through with me to completion. You now know more about leukaemia immunotherapy than you ever thought possible. Thank you for all your generosity of time, patience, dinners, late nights and words of encouragement that have allowed us to see this work in print.

Abstract

Approximately 50% of Acute Myeloid Leukaemia (AML) patients relapse within 5 years of diagnosis. Allogeneic haematopoietic stem cell transplantation (HSCT) has curative potential, partly mediated by a Graft-versus-Leukaemia (GvL) effect. GvL activity may be boosted by donor lymphocyte infusions (DLI) after HSCT, given pre-emptively (pDLI), to prevent relapse in mixed donor chimeric recipients, or therapeutically (tDLI) following disease recurrence. Few publications report efficacy of these approaches, therefore a retrospective analysis of outcomes after DLI at our institution, following lymphodepleted, reduced intensity conditioned HSCT for AML/myelodysplastic syndromes (MDS), was performed. Encouraging estimated 5-year overall survival rates following DLI of 80% for pDLI recipients and 40% for tDLI recipients were observed. Incidence of GvHD was only moderate, suggesting delayed add-back of immune cells can boost GvL reactions in AML/MDS patients without excessive toxicity. However, despite association of DLI with reduced relapse, leukaemia recurrence in a proportion of patients highlights that GvL activity is neither guaranteed nor universally sustained.

Two forms of vaccination are described in this thesis, aiming to enhance priming and activity of leukaemia-reactive T-cells. Both offer broad applicability across all human leucocyte antigen (HLA)-types. T-cell responses to peptide vaccinations targeting the leukaemia-associated antigen Wilms' Tumour protein (WT1) were enhanced by exploration of novel adjuvants for induction of cell-mediated immunity. Vaccinations comprising these adjuvants and single peptides or overlapping peptides spanning the whole WT1 protein, induced functional T-cell responses (antigen-specific *in vivo* cytolytic activity and interferon-gamma production) in C57BL/6 mice. Secondly, autologous AML blasts, genetically modified to express the immunostimulatory molecules CD80 and IL-2, were co-administered as a vaccine with tDLI in a Phase I clinical trial. Preliminary data supporting safety of this approach and possible induction of immune responses to vaccination, evidenced by development of a delayed-type hypersensitivity reaction in a subject, are presented.

This thesis describes broad-based immunotherapeutic strategies in AML patients for prevention of disease recurrence by enhancement of anti-leukaemic immune responses.

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List of Abbreviations

1-MT	1-methyl trytophan
ABL1	Abelson murine leukemia viral oncogene homolog 1
ACV	AML Cell Vaccine
Ag	Antigen
ALC	Absolute Lymphocyte Count
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
ANOVA	Analysis of variance between groups
APC	Allophycocyanin
APC	Antigen Presenting Cell
ANN	Artificial Neural Network
ASC	Apoptosis-associated speck-like protein containing a CARD (caspase-recruitment domain)
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
ATG	Anti-thymocyte Globulin
ATP	Adenosine triphosphate
BAGE	B-Melanoma antigen
BCG-CWS	Bacille Calmette Guerin – Cell Wall Skeleton
BCL-2	B-Cell Lymphoma 2
BD	Beckton Dickinson
BLCL	B-lymphoblastoid Cell Line
BM	Bone Marrow
BMI1	B lymphoma Mo-MLV insertion region 1 homolog
BSU	Biological Services Unit
CAR	Chimeric Antigen Receptor
CASAC	Combined Adjuvants for Synergistic Activation of Cellular Immunity
CCR/CXCR	Chemokine receptor
CD	Cluster of differentiation
CD40L	CD40 Ligand

cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's Adjuvant
CFSE	Carboxyfluorescein diacetate, succinimidyl ester
CI	Confidence Interval
CLIP	Class II-associated invariant chain self-peptide
CLS	Capillary Leak Syndrome
CML	Chronic Myeloid Leukaemia
CMV	Cytomegalovirus
CpG ODN	2'-deoxyribocytidine-phosphate-guanosine oligodeoxynucleotides
cppt	Central polypurine tract
CR	Complete Remission
CRS	Cytokine release syndrome
cts	Central termination sequence
CTCAE	Common Toxicity Criteria of Adverse Events
CTL	Cytotoxic T-Lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DEAE	Diethylaminoethyl
DC	Dendritic cell (pDC, plasmacytoid; cDC, conventional)
dH_20	Distilled water
DLI	Donor Lymphocyte Infusion
DLT	Dose-limiting toxicity
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNAM1	DNAX accessory Molecule-1
DTH	Delayed Type Hypersensitivity
EBV	Epstein-Barr Virus
ECOG	Eastern Cooperative Oncology Group
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated Glomerular Filtration Rate
EGR	Early Growth Response Receptor
eh/pr	Enhancer/promoter
ELISA	Enzyme-linked Immunosorbent Assay
ELISpot	Enzyme-linked Immunosorbent spot assay

ER	Endoplasmic Reticulum
ERAAP	ER aminopeptidase associated with antigen presentation
FACS	Fluorescence-activated cell sorting
FBC	Fludarabine-Busulphan-Campath (Alemtuzumab)
FBL-3	Friend's Leukaemia Virus-induced Leukaemia
FBS	Fetal Bovine Serum
FDC	Full donor chimerism
FITC	Fluorescein isothiocyanate
Flt-3	Fms-like tyrosine kinase receptor-3
FoxP3	Forkhead box P3
FSC	Forward scatter
FU	Follow-up
G250	Renal Cell Carcinoma-Associated Antigen G250, also known as Carbonic Anhydrase IX
GAG	Group-specific antigen
gDNA	Genomic DNA
GITRL	Glucocorticoid-induced-TNFR-related protein ligand
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GMP	Good Manufacturing Practice
GvH	Graft-versus-Host
GvHD	Graft-versus-Host Disease (aGvHD, acute GvHD; cGvHD, chronic GvHD)
GvL	Graft versus Leukaemia
h-TERT	Human Telomerase Reverse Transcriptase
HAGE	Helicase antigen
her2	Human Epidermal Growth Factor Receptor 2
HLA	Human leukocyte Antigen
HOX	Homeobox
HSCT	Haematopoietic Stem Cell Transplant/Transplantation
HPV	Human Papilloma Virus
HTLV	Human T-lymphotropic Virus
HTS	High-throughput sampler
ICAM-1	Intercellular Adhesion Molecule-1
i.d.	Intra-dermal
IDO	Indoleamine 2,3 dioxygenase

IEDB	Immune epitope database
IFA	Incomplete Freund's Adjuvant
IFN	Interferon
Ig	Immunoglobulin
IGF®	Insulin-like Growth Factor (Receptor)
IκB	inhibitor of NF-κB
IL	Interleukin
IMPD	Investigational Medicinal Product Dossier
i.p.	intraperitoneal
IPAF	ICE-protease-activating factor
IRF	IFN-regulatory factor
i.v.	Intravenous
KIR	Killer-cell Immunoglobulin-like Receptor
KLH	Keyhole Limpet Haemocyanin
LAA	Leukaemia Associated Antigen
LCMV	Lymphochoriomeningitis Virus
LPS	Lipopolysaccharide
LSA	Leukaemia-specific antigen
LSC	Leukaemic Stem Cell
LTR	Long terminal repeat
LV_AML	Lentiviral-modified AML
MA	Myeloablative
MAGE	Melanoma-associated Antigen
MAIMP	Manufacturer's Authorisation for Investigational Medicinal Products
MAP	Mitogen activated protein
MCB	Master Cell Bank
Mcl-1	Myeloid cell leukemia sequence 1
MDA5	Melanoma-differentiation-associated Gene 5
MDC	Mixed Donor Chimerism
MDS	Myelodysplastic Syndrome
MDSC	Myeloid Derived Suppressor Cells
MFI	Mean fluorescence intensity
mHag	Minor histocompatibility antigen

MHC	Major Histocompatibility Complex
MICA/B	MHC Class I chain-related gene A/B products
MNC	Mononuclear cell
MOI	Multiplicity of Infection
MPL	Monophosphoryl Lipid A
MPN	Myeloproliferative neoplasm
MPP11	M-phase phosphoprotein 11
mRNA	Messenger ribonucleic acid
MRD	Minimal residual disease
MSC	Mesenchymal Stem Cell
MTD	Maximum Tolerated Dose
MUC1	Mucin1
MyD88	Myeloid differentiation primary-response gene 88
NALP	NACHT-, LRR- and pyrin-domain-containing protein
NCI	National Cancer Institute
NCR	Natural Cytotoxicity Receptor
NCBI	National Centre for Biotechnology Information
NF-κB	Nuclear Factor kappa B
NH_4Cl	Ammonium chloride
NK	Natural Killer
NKG2D	Natural killer group 2, member D
NOD	Non-obese diabetic
NOD	nucleotide-binding oligomerization domain
NPM1	Nucleophosmin 1
NRM	Non-relapse mortality
NUP214	Nucleoporin 214 kDa
ODN	Oligodeoxynucleotide
OS	Overall Survival
O/W	Oil-in-water
OFA-iLRP	Oncofetal antigen/immature laminin receptor protein
OVA	Ovalbumin
OVA-ISQ	ISQAVHAAHAEINEAGR
OVA-SIINF	SIINFEKL

PADRE	Pan-HLA-DR-epitope
PAMP	Pathogen associated molecular pattern
PB	Peripheral blood
PBL	Peripheral Blood Lymphocyte
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD1/PDL1	Programmed Death receptor 1/Programmed Death Receptor 1 Ligand
PDGF(R)	Platelet-derived Growth Factor (Receptor)
pDLI	Pre-emptive donor lymphocyte infusions
PE	Phycoerythin
PerCP	Peridinin-chlorophyll-protein complex
PFS	Progression-free survival
PI	Principal investigator
PI3	Phosphoinositide-3 kinase
PMA	Phorbol 12-myristate 13-acetate
pMHC	peptide/MHC
PML	promyelocytic leukaemia
Poly I:C	Polyinosinic–polycytidylic acid
PR1	Proteinase-3 (PR3) derived antigen
PRAME	Preferentially expressed antigen in melanoma
PRR	Pattern recognition receptor
qPCR	Quantitative PCR
RA(RS)	Refractory anaemia (with ring sideroblasts)
RAEB	Refractory anaemia with excess of blasts
RAG2	Recombinase Activating Gene 2
RARA	Retinoic acid receptor alpha
RBC	Red Blood Cell
RCL	Replication Competent Lentivirus
REV	Regulator of virion expression
RHAMM	Hyaluronan-mediated motility receptor
rHu	Recombinant Human

RIC	Reduced Intensity Conditioning
RICK	Receptor-interacting serine/threonine kinase
RIG-I	Retinoic-acid-inducible gene I
RRE	Rev response element
RT	Reverse transcriptase
RT-qPCR	Real-time qPCR
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RRE	Rev-responsive element
RS	Ring sideroblasts
RUNX1	Runt-related transcription factor 1
RUNX1T1	Runt-related transcription factor 1; translocated to 1, (cyclin D-related)
s.c.	Sub-cutaneous
SCF	Stem Cell Factor
SCID	Severe Combined Immunodeficiency
SD	Standard deviation
SFFV	Spleen focus-forming virus
SLP	Synthetic Long Peptide
SSC	Side scatter
STR	Short tandem repeat
SYK	Spleen tyrosine kinase
TAA	Tumour-associated antigen
TAP	Transporter associated with antigen processing
TBK1	TANK-binding kinase 1
TCD	T-cell depletion
TCR	T-cell Receptor
tDLI	Therapeutic donor lymphocyte infusions
TGF	Transforming Growth Factor
Th	T-helper
TIR	Toll/IL-1R domain
TIRAP	TIR domain-containing adaptor protein
TIL	Tumour-infiltrating lymphocyte
TLR	Toll-like Receptor

TNF	Tumour Necrosis Factor
TRAIL	TNF-related apoptosis inducing ligand
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T-cell
TRIF	TIR-domain-containing adaptor protein inducing IFNβ
TRP-2	Tyrosinase protein 2
UPN	Unique patient number
v/v	Volume per volume
VIN	Vulval intra-epithelial neoplasia
VSV-G	Vesicular stomatitis virus glycoprotein
VUD	Volunteer unrelated donor
WCBP	Women of childbearing potential
W/T	Wild type
WT1	Wilms' Tumour 1
WT1-PGC	PGCNKRYFKLSHLQMHSRKHTG
WT1-RMF	RMFPNAPYL
WT1-YMF	YMFPNAPYL

Chapter 1 Introduction

1.1 Current outcomes for patients diagnosed with Acute Myeloid Leukaemia

Acute Myeloid Leukaemia (AML) is a haematological neoplasm with an age-adjusted incidence of approximately 3 per 100,000 persons/year, the median age at diagnosis being 65. It is characterised by an abnormal clonal proliferation of primitive myeloid cells[1]. Typical morphologic appearances, immunophenotypic marker expression and recurrent genetic changes allow the disease to be classified into defined subtypes (Appendix A)[1].

Currently, therapy for AML is limited to chemotherapy to induce remission, followed by further chemotherapy or allogeneic haematopoietic stem cell transplantation (HSCT) as consolidation to prevent relapse. Prognostic variables, such as increasing age, history of antecedent bone marrow disorder and presence (as well as number) of specific karyotypic and molecular abnormalities, allow stratification of patients according to the risk of relapse[2]. Large randomised controlled studies have identified those patients with good cytogenetic/molecular risk profiles suitable for treatment with chemotherapy alone, versus those with adverse cytogenetic/molecular risk profiles for whom allogeneic HSCT as consolidation offers the only chance of long-term cure[2]. The greatest difficulty in deciding the optimal consolidation strategy after first remission applies to the large population of patients with normal karyotype AML[2,3]. Recent advances in understanding the prognostic implications of AML-associated genetic aberrations in the context of normal karyotype AML, such as mutations in nucleophosmin 1 (*NPM1*) and FMS-like tyrosine kinase 3 (*Flt-3*) genes, have improved risk stratification in these patients[4]. Prognostication for this cohort of patients with heterogeneous outcomes is likely to improve as genomic sequencing data from large cohorts of AML patients, combining details of additional gene mutations, accumulates[5].

In recent decades there has been an overall improvement in survival principally for patients under the age of 60 at diagnosis of AML (see Figure 1-1)[2,6]. Better outcomes have been attributed to superior supportive care strategies, such as infection prophylaxis, and the selected use of allogeneic haematopoietic stem cell transplantation (HSCT) in appropriate patients. By contrast, there have been few advances in the cytotoxic

agents used for remission induction and consolidation, which have remained largely unchanged during the same time period.

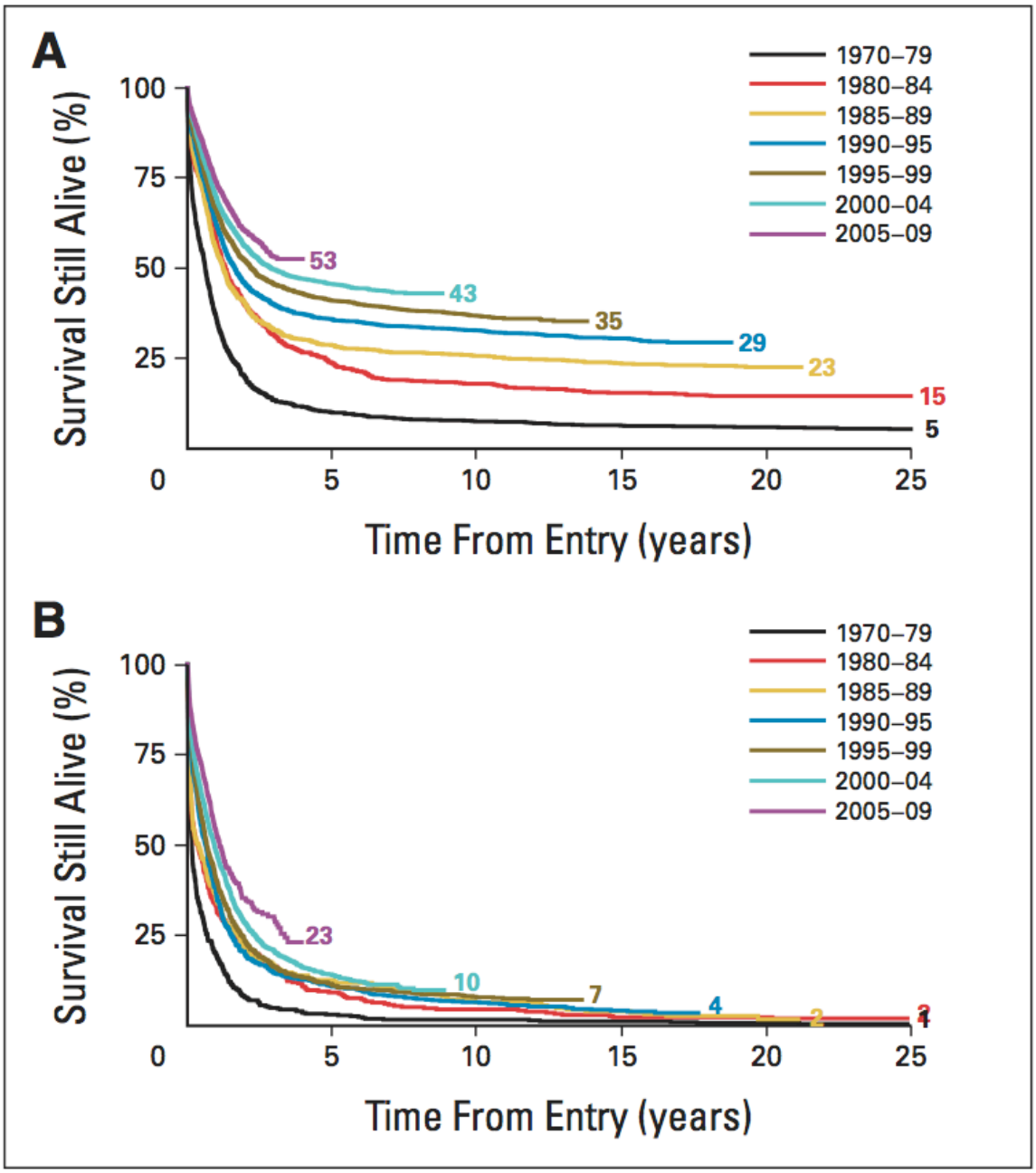


Figure 1-1 Changes in survival for AML patients aged 15-59 years (A) or over 60 (B) at diagnosis over the last four decades. Taken from Burnett et al,[6].

In spite of these improvements, while up to 80% of AML patients may enter a period of remission following initial induction chemotherapy, the risk of relapse overall is greater than 50% within the first 2 years[3,7,8]. The poorest outcomes are observed in the large proportion of patients aged over 60 at diagnosis of AML, with less than a quarter of these patients surviving to 5 years (Figure 1-1)[6]. This adverse prognosis reflects both disease biology (higher incidence of adverse prognostic factors in older patients) and also increased comorbidity, limiting the intensity (and efficacy) of cytotoxic agents used[9]. Given the higher risk of relapse in older patients, HSCT offers the best chance of cure for this group. However, the organ toxicity of standard myeloablative (MA) transplant conditioning regimens, arising from both the cytotoxic drugs and early, severe Graft-versus-Host (GvH) reactions, has been associated with prohibitive non-relapse mortality (NRM) in these patients. Demonstration of the dominant role that donor lymphocytes play in mediating a graft-versus-leukaemic (GvL) effect that maintains remissions post-HSCT[10-12] led to the development of reduced intensity conditioning (RIC) regimens. These regimens have an acceptable NRM in older patients (approximately 20-30% at 5 years) due to the less cytotoxic and more immunosuppressive nature of the agents used for conditioning, reducing organ damage and incidence of severe acute graft-versus-host disease (GvHD)[13,14]. RIC aims to exploit GvL activity due to allorecognition of tumour cells by donor-derived lymphocytes as the principal means of disease control. Furthermore, HSCT has the potential to serve as an immunotherapeutic platform, whereby the GvL effect arising from the graft can be subsequently enhanced, for example by add-back of donor lymphocytes. However, the weaker cytotoxicity of RIC regimens, combined with delayed immune reconstitution observed in athymic older HSCT recipients has been associated with a persisting risk of relapse in retrospective comparisons[15-17]. The additional use of T-cell depletion (TCD) in conditioning regimens to prevent graft rejection and attenuate GvHD may also hinder immune reconstitution, with an attendant risk of relapse[18,19]. Once AML recurs post-HSCT, salvage using chemotherapy alone or in combination with donor lymphocyte infusions (DLI) has been associated with dismal rates of overall survival approximating 20% or less at 2 years[20,21].

Clearly newer treatment strategies to advance the therapy of AML are desperately required. Despite many years of research, front-line cytotoxics used to treat AML have not changed, highlighting the limited impact of novel agents[2,6]. Immunotherapeutic approaches to the treatment of AML are highly attractive, offering the possibility of targeted eradication of leukaemic cells not only in HSCT patients but also in

those patients deemed unsuitable for HSCT[22,23]. Adjuvant immunotherapy may be one means to induce and maintain GvL responses after stem cell transplantation[24,25]. The following sections describe the evidence for immune mediated-eradication of AML, providing a rationale for AML immunotherapy and discussing barriers to the success of such treatment. Active (immunisation) and passive (adoptive cell transfer, e.g. DLI) strategies promoting anti-leukaemic immune responses within patients are described. Vaccination approaches receive a particular emphasis as a background to the presented work in this thesis evaluating peptide and whole cell immunisation targeting AML.

1.2 Evidence for immune surveillance of AML

Scientists such as Ehrlich, Burnet and Thomas originally hypothesised that the immune system is capable of recognising and specifically eliminating tumour cells arising within an organism. They proposed that immune cells constantly survey for malignant cells and act to either repress their growth or induce death[26]. The development of sophisticated immunodeficient mouse models delineated relative contributions of different components of the immune system to this process. Mice lacking interferon gamma (IFNγ) responsiveness (due to absent/non-functional IFNγ receptor or downstream signalling components) or deficient in B, T and NKT-cells (recombination activating gene 2 [RAG2] knockout mice) demonstrate greater susceptibility to spontaneous and induced solid tumours[26-29]. Other cell types, such as γδ T-cells[30,31] and Natural Killer (NK cells)[32], and molecules, such as perforin[33], tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL)[34], have all been shown to restrict tumour growth when present and functional. Similarly, in human pathology, immunosuppressive therapy or inherited defects of the immune system are associated with increased risk of malignancies[28].

In the specific setting of AML, several lines of evidence support the role of various components of the immune system in surveillance and eradication of leukaemic blasts. Multivariate analysis has shown that superior absolute lymphocyte count (ALC) recovery at specific time points post induction chemotherapy is significantly associated with improved overall and leukaemia-free survival in patients with AML or acute lymphoblastic leukaemia (ALL)[35,36]. Furthermore, studies have demonstrated that NK-[37,38], T[39]-cells and more recently, NKT[40] and γδ T-cells[41] isolated from patients in remission post chemotherapy for AML can

lyse autologous primary blasts. The ability of DLI to induce remission in patients with recurrent chronic myeloid leukaemia (CML) following allogeneic HSCT has provided some of the strongest evidence for an immune-mediated destruction of leukaemic cells *in vivo*[10,11]. In the context of fully Human Leukocyte Antigen (HLA)-matched allogeneic HSCT, these GvL reactions have been demonstrated to arise through donor T-cell allorecognition of epitopes from mismatched minor histocompatibility antigens (mHags), presented in the context of major histocompatibility complex (MHC) molecules and showing higher expression on leukaemic cells than healthy tissue. Responses against mismatched mHags expressed by healthy recipient tissues may however result in unwanted GvHD[42].

Leukaemia-specific T-cell responses also occur in the autologous, non-HSCT setting, whereby lymphocytes recognise epitopes derived typically from self antigens showing aberrantly high expression in tumour cells. Peptide elution studies to isolate epitopes bound to MHC molecules on tumour targets and complementary deoxyribonucleic acid (cDNA) screening libraries produced from tumour cells provided the initial means to identify immunogenic tumour-associated antigens (TAAs) in the context of tumours such as melanoma[43]. As knowledge of peptide/MHC (pMHC) binding and T-cell receptor (TCR) recognition has advanced, *in silico* prediction tools have accelerated the identification of immune epitopes from a wide range of TAAs[44].

A number of leukaemia-associated antigens (LAAs) expressed by primary AML blasts are recognised (Table 1). Cytotoxic T lymphocytes (CTLs) have been isolated from AML patients and demonstrated *in vitro* to respond to the specific LAA-derived peptide in an MHC-restricted manner, resulting in IFNγ production or target cell lysis[45,46]. In addition, serological responses to LAAs have been detected in patients with myeloid diseases whilst remaining undetectable in healthy volunteers, suggesting activation of B-cells and helper T-cells[46]. Occasionally, uniquely expressed leukaemia-specific antigens (LSA) may be the targets of T-cell responses. Examples include epitopes arising from fusion proteins such as RUNX1-RUNX1T1[47], PML-RARA[48] or DEK-NUP214[*49] resulting from specific gene translocations [t(8;21), t(15;17) and t(6;9) respectively]. The low frequency of these specific translocations within AML patients (5%, 5-8% and <2% respectively) restricts their potential as therapeutic targets[1]. Mutations in *Flt-3* and *NPM1* genes, in up to

[*] *RUNX1,* Runt-related transcription factor 1; *RUNX1T1*, Runt-related transcription factor 1, translocated to 1, (cyclin D-related); *PML,* promyelocytic leukaemia; *RARA*, retinoic acid receptor alpha; NUP214, nucleoporin 214kDa

40% and approximately 30% of patients with AML respectively, are more frequently observed[1]. T-cell responses directed at epitopes derived from these mutant proteins have also been documented[50-52].

The role that chemotherapy plays in the induction of tumour-specific immune responses is also noteworthy[53,54]. Chemotherapy reduces tumour bulk, but in some experimental models, it has been shown that apoptosing neoplastic cells stimulate immune cells[54,55]. In combination, reduction of tumour load and activation of tumour-reactive immunity may allow the immune system to regain control over neoplastic expansion[53,54]. Lymphodepleting chemotherapy agents, such as fludarabine and cyclophosphamide may be used to influence adaptive immunity through different mechanisms[53]. Lymphopenia-induced homeostatic expansion of adoptively transferred tumour-specific cytotoxic T-cells occurs after fludarabine and cyclophosphamide treatment[56]. Immunomodulating low doses of cyclophosphamide deplete regulatory T-cells (Tregs) that normally act to limit expansion of high avidity, self-antigen recognising T-cells. Depletion of Tregs by prior low dose cyclophosphamide treatment has been shown to allow effective induction and expansion of tumour-specific (self-reactive) T-cell responses following peptide vaccination against a particular TAA[57]. Newer treatments for AML, which also show immunomodulatory activity, such as the demethylating agent 5-azacytidine, have been reported to induce tumour-specific T-cell responses. Evidence from *in vitro* studies suggested that 5-azacytidine promotes upregulation of epigenetically silenced tumour associated antigens (TAAs) by tumour cells, allowing enhanced antigen presentation and activation of tumour specific CTLs[58].

Another area of emerging interest is that anti-leukaemic immune responses may be effective against leukaemic stem cells (LSC)[59]. Chemotherapy resistance of LSCs has been postulated as a mechanism for leukaemic relapse[60]. The largely quiescent nature of these cells may protect them from apoptosis induction upon exposure to cytotoxics[60]. Whether long-term disease control may arise from antigen-specific T-cells targeting chemotherapy-resistant LSCs directly, or rather their progeny, is an area of active research interest[22,60]. Immune therapies may be more effective than cytotoxic agents against LSCs, due to their cell-cycle independent mechanism of action.

The evidence above supports the role of the immune system in detecting and eradicating AML. However, this is clearly failing at the time of disease presentation or relapse. Strategies by which AML may evade the immune system are described in the next section.

Table 1-1 Expression of candidate leukaemia associated antigens in AML, normal tissues and normal CD34+ cells – adapted from Greiner et al[46] and Anguille et al[50]

Leukaemia associated antigen*	Function	% of AML patients showing LAA over-expression in AML cells	Expression in normal tissue	Expression in normal CD34+ BM cells
BAGE	Unknown	27%[61]	Restricted to germ cells[61,68]	Not expressed[61]
BCL-2	Apoptosis regulation	84%[75]	Various normal tissues[69]	Expressed[62]
CML28	Processing, degradation of RNA	89%[70]	Not expressed outside of testis[70]	Expressed[63]
G250	Hypoxia regulated gene	51%[61]	Not expressed[61,71]	Not expressed[61]
HAGE	Unknown	23%[72]	Testis, placenta, pancreas[72]	Unknown
hTERT	Catalytic subunit of the telomerase gene	28%[61]	Not expressed[61]	Not expressed[61]
MPP11	Cell proliferation	86%[61]	Testis, kidney, lung[61]	Expressed[61]
OFA/iLRP	Receptor for prion protein uptake into cells	100%[64]	Not expressed[64]	Not expressed[64]
PRAME	Induction of apoptosis, possible repressor of retinoic acid receptor signalling	64%[61]	Testis, placenta, ovary[61]	Not expressed[61]
PR3	Neutrophil serine elastase, IL-32 binding protein	67%[61]	Myeloid cells and other normal tissues[61]	Expressed[61]
RHAMM	Mitotic spindle formation, signal transduction	70%[61]	Testis, thymus, placenta[61]	Not expressed[61]
Survivin	Inhibition of apoptosis	100%[76]	Various normal tissues including haematopoietic cells[73]	Expressed[65]
WT-1	Transcription factor	67-86%[61,67]	Placenta, kidneys, gonads, thymus, spleen[74]	Expressed[66,67]

*For a full explanation of abbreviations, please refer to the main Abbreviations section.

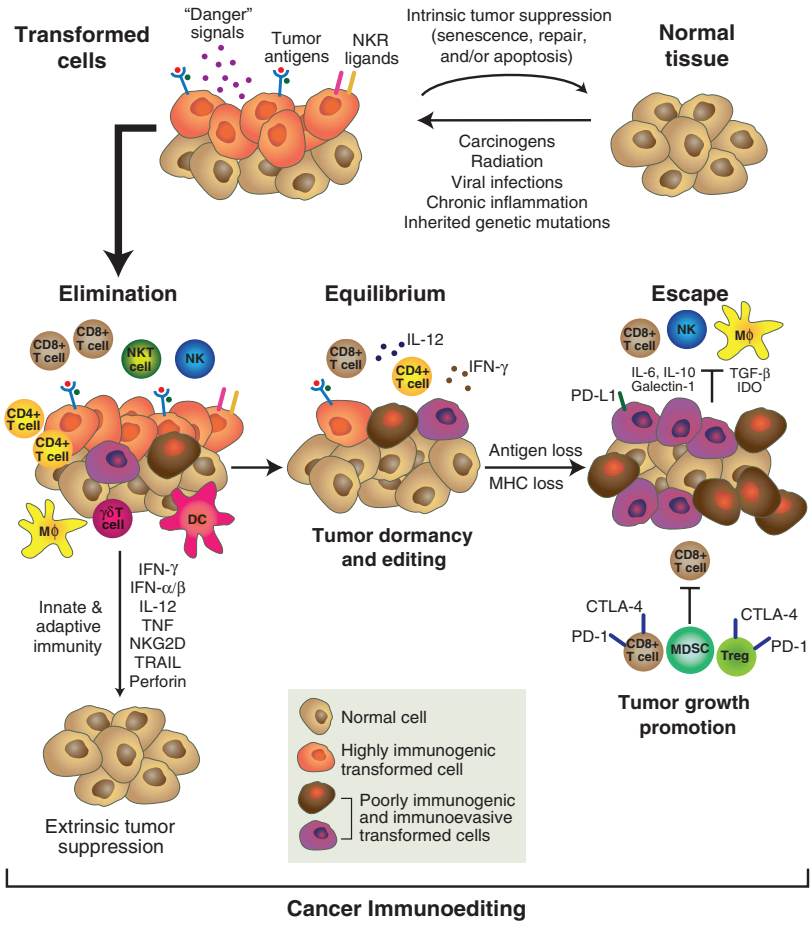
Table 1-1 continued

Leukaemia associated antigen	Function	% of AML patients showing LAA over-expression in AML cells	Expression in normal tissue	Expression in normal CD34+ BM cells
BMI1	Modulation of haematopoietic stem cell self-renewal	55%[89]	Various normal tissues[77]	Expressed[77]
Cyclin A1	Cell cycle regulator	50%[78]	Testis[84]	Not expressed[78]
Cyclin B1	Cell cycle regulator	98%[90]	Various normal tissues[85]	Expressed[79]
HOXA9	Embryonic development, haematopoiesis	68%[91]	Various normal tissue [86]	Expressed[80]
Mcl-1	Apoptosis regulator	100%[81]	Various normal tissues[87]	Expressed[81]
Mesothelin	Possible adhesion molecule	81%[92]	Pleura, peritoneum, pericardium[82]	Not expressed[82]
MUC1	Lubrication of epithelium	67%[93]	Glandular epithelium[88]	Expressed[83]

1.3 Immune evasion by AML

Despite the potential for the immune system to detect and destroy leukaemic cells, the neoplastic process ultimately subverts this, allowing disease progression or recurrence. A process of dynamic shaping of immune responses and tumour immunogenicity favours disease control or relapse, according to the immunoediting hypothesis proposed by Schreiber. Three distinct phases in the relationship between a tumour and the immune system have been suggested, namely elimination, equilibrium and escape (Figure 1-2)[29]. In the earliest stages of malignant disease, immunogenic tumour cells are highly vulnerable to destruction by the innate and adaptive immune systems, preventing clinically evident malignancy. Less immunogenic tumour cells survive the elimination phase and persist during equilibrium, ultimately leading to clinically evident disease (escape). A long-term equilibrium on the other hand is manifested by sustained remission[29].

Figure 1-2 The cancer immunoediting hypothesis



MΦ: Macrophage

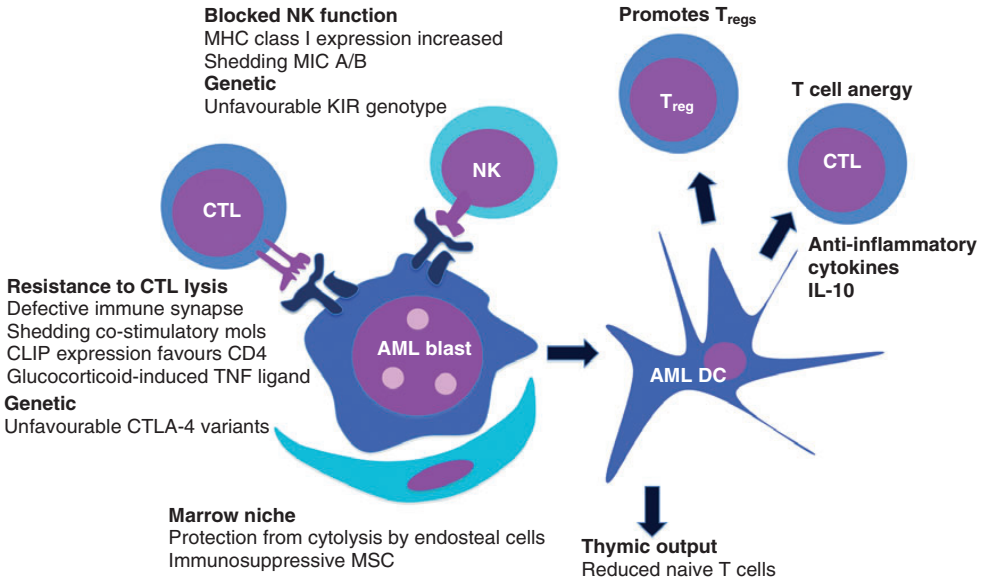
DC: Dendritic cell

MDSC: Myeloid derived suppressor cell

Taken from Schreiber et al,[29]. See the main Abbreviations list for a full explanation of abbreviations.

AML cells have been observed to employ a number of mechanisms in order to evade immune recognition and destruction (Figure 1-3).

Figure 1-3 Mechanisms of immune evasion by AML blasts



Taken from Barrett and Le Blanc [22]. See the main Abbreviations list for a full explanation of abbreviations.

Immune evasion may involve avoidance of T- or NK-cell recognition, or promotion of dysfunctional/inhibitory immune responses by AML blasts. NK cells express a number of activating receptors, including the natural cytotoxicity receptors (NCRs, NKp44, NKp46 and NKp30) or DNAX accessory Molecule-1 (DNAM-1) and Natural killer group 2, member D (NKG2D)[94]. Down-regulation of NCRs on NK-cells from AML patients[95] or their ligands on AML blasts has been observed[95,96]. Inhibitory interactions between AML blasts and NK-cells may also occur. Cell surface molecules such as glucocorticoid-induced TNFR-related protein ligand (GITRL)[97] and CD137L[98] expressed by AML blasts bind ligands on NK cells, inhibiting NK cell cytotoxicity and cytokine production. T-cell activation may be avoided by reduced expression of co-stimulatory molecules such as CD80, resulting in failure to deliver necessary co-stimulatory signals and promotion of anergy[99]. Down-regulation of certain HLA Class I alleles by AML blasts, such as those belonging to the HLA Bw6 group that are not sensed by NK cells but present tumour antigens to CTLs, provides another means to specifically evade attack by T-cells[100].

Secretion of immunosuppressive factors by human AML cell lines or primary AML blasts, resulting in inhibition of NK and T-cell proliferation[101] or T-cell function[102] has been previously demonstrated. Elegant studies have demonstrated that T-cells from AML patients at diagnosis were unable to form effective immune synapses with autologous blasts. Further analysis revealed this was due to dysfunction within both the AML blasts and the patients' T-cells[103]. Furthermore, AML blasts derive from myeloid progenitors and therefore may differentiate into dendritic cells (DCs). Studies *in vitro* have suggested that AML-derived DCs show abnormal maturation[104] and promote the generation of suppressive T-regulatory cells (Tregs) through increased expression of indoleamine 2,3-dioxygenase (IDO)[105]. Activated Tregs in turn suppress effector T-cell responses to the tumour.

Natural regulatory mechanisms that operate to terminate T-cell responses may also hinder tumour cell killing. Following engagement of the T-cell receptor (TCR) with a cognate peptide bound to a major histocompatibility complex molecule (pMHC), costimulatory signals are delivered by binding of CD28 to CD80/86 on antigen presenting cells (APCs). Subsequent cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) upregulation by T-cells acts to outcompete CD28 for binding to CD80/86 to terminate the costimulatory signal[106,107]. This results in delivery of inhibitory signals to the T-cells, modulating the amplitude of the response in proportion to the strength of the T-cell activating signal[106,107]. Ligation of constitutively expressed CTLA-4 on Tregs also has a vital physiologic role to allow suppressive control of effector T-cell function[108]. Accordingly, inhibition of CTLA-4 signalling via blocking antibodies has been shown to improve endogenous anti-tumour T-cell responses[109]. Programmed cell death protein 1 (PD-1) acts at a later phase of the T-cell response, to limit effector T-cell activity in the periphery and avoid collateral inflammatory damage to local tissue[106]. Increased expression of PD-1 on tumour infiltrating/specific lymphocytes has been demonstrated[110,111]. Some tumours (including AML[112]) have been shown to up-regulate the PD-1 ligands, PDL1 and PDL2[106,113]. Therefore malignant cells may negatively modulate anti-tumour T-cell (and also PD1-expressing B- and NK-cell) responses, providing an additional means whereby they can evade adaptive immune responses.

There is substantial evidence that immune eradication of leukaemic blasts may contribute to long-term control/cure of the disease. Disease may recur due to the persistence of chemotherapy-resistant leukaemic stem cells within the host. Manipulation of immune responses may be used to enhance eradication of leukaemic blasts as an adjunct to tumour debulking by chemotherapy. Increasing evidence suggests that certain chemotherapeutics may exert some of their effects by eliciting immune responses. Replacement of a "tumour-edited" host immune system in the context of allogeneic transplantation by healthy donor cells capable of GvL activity has curative potential. Boosting of immune responses during clinical remission, within or outside the setting of HSCT, has the potential to target LSCs or their progeny and prevent disease recurrence. Knowledge of the interactions between the immune system and leukaemic cells/progenitors and the mechanisms used to escape from immune surveillance is accumulating. As a result, strategies to beneficially enhance and maintain immune activity against leukaemia and overcome immune escape mechanisms are being explored.

1.4 Immunotherapeutic strategies in AML

A number of approaches have undergone pre-clinical or early phase study to either directly stimulate or passively confer anti-leukaemic immune activity in subjects. Passive mechanisms include adoptive transfer of lymphocytes. At its most basic, this includes the use of donor lymphocyte infusions, described in more detail in section 1.5. Selected lymphocyte subsets may also be transferred, including CD8-depleted DLI[114] or NK cells, derived from haploidentical donors[115]. The development of more sophisticated gene therapy approaches has allowed the generation of antigen-specific T-cells, directed against target LAAs (e.g. TCR gene therapy resulting in the production of WT1-specific T-cells[116]) or T-cells bearing chimeric antigen receptors (CARs) which are not reliant on recognition of pMHC complexes but rather bind to non-polymorphic targets, such as CD123 on AML cells[117].

Active leukaemia-specific immunity is principally induced by vaccination approaches such as whole cell, antigen-loaded DCs, DNA or peptide vaccines targeting the LAAs listed in Table 1-1. Peptide vaccination in a Phase I/II study using the proteinase 3 derived PR1 peptide induced at least a 2-fold expansion in PR1-specific T-cells in nearly half of AML patients vaccinated, with a 4-month increase in progression-free

survival (PFS) in immune responders[118]. Following high dose Hyaluronan-mediated motility receptor (RHAMM) peptide vaccination, Greiner et al demonstrated transient immunological and haematological responses in a mixed cohort of patients with haematological malignancies[119]. Peptide vaccination targeting WT1 has been studied in both pre-clinical and clinical settings and serves as a paradigm of active vaccination approaches to AML; this area is explored in section 1.6. Whole cell vaccination using genetically modified tumour cells (leukaemia cells[120,121] or a leukaemia cell line[122] modified to express granulocyte-macrophage colony-stimulating factor [GM-CSF[122]] or CD80 and IL-2[120,121]) and Phase I/II studies have either been reported[122] or are in progress (RFUSIN2-AML1 vaccine trial, EudraCT Number 2005-000806-29). Whole cell vaccination for AML is described in further detail in section 1.7.

1.5 Donor lymphocyte infusions: adoptive immunotherapy to prevent and treat myeloid malignancies

One of the earliest forms of immunotherapy employed in patients with haematological malignancies has been the use of unmanipulated infusions of donor lymphocytes to treat or prevent recurrent disease post-HSCT. A major breakthrough in the therapy of patients with recurrent chronic myeloid leukaemia (CML) post-allogeneic HSCT was the demonstration by Kolb and co-workers that DLI combined with IFNα therapy could be used to re-induce sustained remissions in these patients[123]. A number of other groups reported efficacy of DLI for therapy of recurrent CML, with high rates of remission seen particularly in patients in early, chronic phase disease at relapse[124-129]. Responses to DLI reported in original case series appeared to coincide in some instances with an increase in donor-derived haemopoiesis and/or the onset of GvHD, or cytopenias due to marrow aplasia, suggesting the induction of alloreactive GvL responses[128,129]. Larger retrospective registry studies evaluated outcomes following therapeutic DLI (tDLI) in a range of other haematological malignancies, including acute and chronic leukaemias, lymphoma and myeloma. The best responses to tDLI were frequently observed in patients with recurrent early phase CML, with detectable but often curtailed responses in the context of relapsed acute leukaemias or accelerated phase CML[11,130].

Specifically in the context of AML and myelodysplastic syndrome (MDS), variable results from retrospective registry studies have been described (Table 1-2). Comparisons between different studies are difficult for a number of reasons. These include the retrospective nature of most studies, use of registry data sourced from multiple institutions, varied reporting of infusion cell type and dose (total nucleated or mononucleated cells, or CD3+ cells, per kg) and the inclusion of heterogeneous disease cohorts comprising small numbers of patients. Further factors confounding the interpretation of results of tDLI have included the treatment of patients with varying pre-therapy levels of disease burden (usually described in terms of leukaemic blast percentage) or the use of an immunomodulatory drug such as IFNα or chemotherapy to reduce disease bulk prior to DLI. Overall, rates of remission induction with tDLI have ranged from 14-47% in the studies listed in Table 1-2 but dismal 2 year overall survival (OS) rates, generally less than 20% at 2 years, have repeatedly been described, largely due to subsequent disease recurrence.

Table 1-2 Outcomes following DLI for relapsed AML and MDS post-HSCT (results from selected series)

Disease type and patient numbers	Number of TNCs, mononuclear or T-cells administered (median and range) $x10^8$ per kg	Numbers receiving induction chemotherapy prior to DLI and number of CRs following chemotherapy	% of patients showing disease response to DLI +/- chemotherapy	Incidence of >Grade 2 acute or chronic GvHD (combined percentage where not specified)	2y Overall Survival (OS) post DLI	Ref
AML, n=23 MDS, n=5	2.4 (0.1-7.83) MNC	n=9, 5 CR	29% AML 25% MDS	41%	15%	Kolb, 1995[11]
AML, n=46 MDS, n=6	6.3 (0.5-14) MNC or 3.2 (3.0-3.5) CD3	n=13, 7 CR	15% AML 40% MDS	46% acute 32% chronic	18%	Collins, 1997[130]
AML, n=32 MDS,n=11	2.2 (0.01-11.3) TNC	Not specified by disease type	28% AML 45% MDS	33% acute 34% chronic	<20%	Shiobara, 2000[131]
AML, n=23	1.34 (0.001-31.8) MNC	n=7, 4 CR	42% (DLI only*)	35% acute 40% chronic	Not given	Porter, 2000[132]
AML, n=47 MDS, n=7	1.0 (0.6 to 4.2) CD3, dose 1 2.35 (1.0 to 6.6) CD3, dose 2	all patients	47% (AML and MDS combined)	56%	19%	Levine, 2002[133]
MDS, n=14	0.6 (0.1-2.8) CD3	n=2, CR=1	14%	50%	Not given	Depil, 2004[134]
MDS, n=16	1.0 (0.1-1.5) CD3	n=3, 2 CR	21%	31% acute 31% chronic	<20%	Campregher, 2007[135]
AML, n=171	0.1 (0.001-2.25) CD3	n=124, 19 CR	34%	43% acute, Grade unknown 23% chronic	21%	Schmid, 2007[136]
AML, n=88	0.1 (no range) CD3	n=48, 10 CR	30% (chemotherapy +DLI recipients) 36% (DLI without chemotherapy)§	Not specified	2y OS from relapse: 12.6% (chemo+DLI) 25% (DLI without prior chemo)	Schmid, 2011[21]

*Patients in complete remission (CR) following chemotherapy were not considered evaluable for response to DLI

§Some of the recipients of DLI without chemotherapy underwent second HSCT, which may also have contributed to the % achieving CR

NRM due to complications such as GvHD (with a reported incidence of acute and/or chronic GvHD in between 20-60% of patients, Table 1-2) also contributes to reduced overall survival (OS). The largest report of tDLI administration for relapsed AML post-HSCT by Schmid et al., a retrospective registry study of 171 recipients, identified factors that were predictive of improved survival response by multivariate analysis. These included presence of fewer than 35% bone marrow blasts at relapse, female gender, favourable cytogenetic disease risk profile and morphological remission at the time of DLI. Overall, 2-year survival following DLI was estimated at 21% ± 3%. This study also reported the outcomes of 228 patients who did not receive any cellular therapy to treat their recurrent disease. Their OS was worse, at 9% ± 2% at 2 years, again suggesting greater prospects for disease control where GvL can be boosted. For the whole cohort studied, relapse beyond 5 months post-HSCT was associated with a superior 2-year OS (27% vs. 13% for those relapsing within 5 months post-HSCT, p=0.0007)[136]. This particular finding may reflect variation in the disease biology between individual patients, influencing the tempo of disease recurrence. It is also possible that those patients who experienced longer remissions post-HSCT had developed some GvL activity, albeit of lessened efficacy around the time of disease recurrence, which could be boosted by fresh infusions of lymphocytes.

Most of the patients described in this study and the others listed in Table 1-2 had received a full intensity and/or T-replete HSCT. There are very few reports of the response to tDLI in patients with AML or MDS following T-cell-depleted, reduced intensity conditioned (TCD RIC) regimens and in general, these patients have been grouped in studies including subjects with myeloid and lymphoid malignancies[137,138]. Shaw and colleagues reported on outcomes for a mixed cohort of patients following TCD RIC HSCT, given DLI for a variety of indications, including recurrent disease[139]. They demonstrated a 42% complete response and 19% partial response to DLI in 36 recipients with persistent or relapsed disease post-TCD RIC HSCT[139]. Early reports of outcomes from our institution of tDLI for morphologic or cytogenetic relapse of MDS/AML post fludarabine, busulphan and Campath® (alemtuzumab) (FBC)-conditioned allograft in small numbers of patients demonstrated limited response to DLI following morphological evidence of disease recurrence, but complete remissions in over half of patients treated for cytogenetic relapse only[13,140]. Given the lack of data to demonstrate efficacy for the use of tDLI for relapsed MDS/AML post-TCD RIC allografts, one aim of this thesis was to examine outcome data from our institution in more detail. This is especially relevant

in the context of our Phase I clinical trial of whole cell vaccination using CD80/IL-2 modified AML blasts, which combines vaccination with administration of tDLI to improve the efficacy of this form of adoptive immunotherapy.

The use of pre-emptive DLI (pDLI) to prevent relapse following TCD RIC HSCT for AML/MDS is another area where there are limited data. Furthermore, centres may report outcomes following more than one indication for administration of DLI in the absence of overt relapse: planned, prophylactic administration of DLI early post-HSCT in high-risk patients, versus administration guided by a persistently low, or falling, donor T-cell chimerism. Within our institution, pDLI has been given to patients with persistent predominant recipient T-cell chimerism or a progressive fall in donor CD3+ percentage on successive readings[13,140,141]. Therefore, the term pDLI will be used in this thesis to describe only this indication.

Mixed donor chimerism (MDC) is often reported following TCD and/or RIC HSCT, in contrast to myeloablative, non T-cell depleted HSCT[139,142-146]. The prognostic significance of persistent MDC following TCD RIC HSCT remains an area of ongoing debate. Whilst some studies have reported MDC to be associated with an increased risk of relapse[139], others have linked MDC to lower rates of GvHD and no demonstration of higher disease recurrence rates[147]. In part, these differences arise due to the variety of diseases, HSCT conditioning regimens and choice (as well as dose) of T-cell depleting antibody used in the studies. Furthermore changes over time in the technologies used to measure chimerism (e.g. karyotyping versus quantitative polymerase chain reaction (qPCR) of short tandem repeat (STR) sequences), the use of whole bone marrow or blood, or lineage-specific analyses of peripheral blood subsets, and a lack of uniform criteria to define MDC, confound comparisons between groups. To counter some of these issues, consensus definitions and approaches to measurement of chimerism have been suggested[148,149].

Specifically, whether reduced donor T-cell chimerism predicts increased likelihood of relapse in the T-deplete transplant setting is controversial. Van Besien et al[143] and Nikolousis et al[150] studied heterogeneous cohorts of patients who underwent RIC HSCT incorporating the lymphocyte-depleting anti-CD52 antibody alemtuzumab. Neither reported a significant impact of the presence of mixed T-cell

chimerism before day 180 on relapse risk. However, Van Besien and colleagues did demonstrate that a successive decline in CD3 chimerism of more than 15% between days 30-180 was predictive of a 40% risk of relapse, in comparison with a 10% risk of relapse amongst those with stable CD3% between these time points[143]. Where anti-thymocyte globulin (ATG) was used for T-cell depletion, Mohty and colleagues reported a significantly higher risk of relapse in patients following RIC HSCT for myeloid malignancies showing predominant recipient T-cell chimerism at day 90[151]. Mattsson and colleagues included a selection of patients in their report who received ATG as part of RIC and found no association between relapse risk and mixed T-cell chimerism, although this study included children and adults with both malignant and non-malignant diseases[146].

Given indications that declining donor T-cell chimerism may portend relapse, a number of groups have sought to explore the ability of pDLI to convert mixed to full donor T-cell chimerism and assess impact on disease-free survival and measure toxicity, such as development of GvHD. Pre-emptive DLI has been shown to effectively restore full donor chimerism (FDC) following alemtuzumab-containing RIC in more than half of mixed chimeric patients with Hodgkin's[144] and non-Hodgkin's lymphoma[152] patients and a range of other haematological malignancies[142,153]. Peggs et al were also able to demonstrate a very low rate of recurrent Hodgkin's disease and survival advantage for recipients of pDLI for mixed chimerism over patients spontaneously achieving FDC[144]. Rates of acute and/or chronic GvHD following pDLI in these studies ranged from 20-40% with DLI-related mortality approaching 10% of recipients at worst[142,144,153]. pDLI was not administered to these patients before 6 months post-HSCT, based on earlier retrospective studies suggesting a >50% chance of acute or chronic GvHD induction where DLI was administered earlier than 6 months post-HSCT[137]. Furthermore, an escalating rather than bulk dose schedule for administration of donor lymphocytes was used as a means to attenuate toxicity[153,154], although dose increments and intervals have not been evaluated prospectively in a randomised manner.

These studies have therefore shown effective and durable response to pDLI particularly in the context of lymphoma. Importantly, conversion to FDC and durable remissions were not always accompanied by clinical evidence of GvHD, suggesting that it is possible to modulate chimerism in such a way as to

promote alloreactive graft-versus-lymphoma responses without toxicity. The efficacy of pDLI for haematological malignancies that tend to show a more rapid pace of recurrence, such as AML, has been less clearly documented. Given that AML frequently recurs during the first 6 months following TCD RIC HSCT[155], there is also a rationale for earlier intervention with pDLI to prevent relapse. A retrospective analysis from our institution has shown that around half of patients who underwent TCD RIC HSCT for MDS, or AML secondary to MDS, have mixed donor T-cell chimerism at 6 months to 1 year[141]. Lim et al demonstrated that 17/28 (61%) patients following FBC RIC HSCT for MDS/AML converted from MDC to FDC after receiving pDLI. Of the 28 recipients of pDLI, 10 (36%) subsequently developed GvHD[156]. The median time to pDLI post-HSCT was 196 days (range 76–469 days) and a median of 2 doses of escalating dose pDLI were given (range 1-7). In the small cohort of pDLI recipients patients described, median OS and disease-free survival (DFS) of 80% and 77% respectively at 2 years were observed. Thus these encouraging findings support potential efficacy of pDLI in patients with predominant recipient or declining donor T-cell chimerism but an assessment of efficacy and toxicity in a larger cohort of patients is warranted and forms part of this thesis.

1.6 WT1 peptide vaccination targeting AML

A recent pilot project by the National Cancer Institute identified the WT1 protein as the highest priority TAA to be considered when designing studies of immunotherapy for malignant disease[157]. This was based on a number of criteria, including (1) specificity of expression by cancer cells, (2) expression levels, (3) surface or intracellular localization within tumour cells, (4) frequency of over-expression within patients, (5) oncogenicity and (6) evidence of immunogenicity *in vivo*[157]. Although no candidate TAA possessed all desirable characteristics, WT1 surpassed other LAAs according to these criteria. WT1 remains the most extensively studied LAA to date in the context of leukaemia immunotherapy. Its suitability as a target and utility in peptide vaccines for leukaemia immunotherapy is outlined below, with detailed discussion of peptide vaccination studies targeting WT1 to date.

1.6.1 Suitability of WT1 as a target for immunotherapy of AML

The Wilms' Tumour (*WT1*) gene, located on chromosome 11p13, was identified as a tumour suppressor in cases of sporadic and hereditary paediatric nephroblastoma[158]. The gene spans 50Kb and is comprised of 10 exons containing alternative splice sites resulting in the production of 4 protein isoforms[159,160]. The protein product is a member of the Early Growth Response (EGR) family of transcription factors, possessing four zinc finger motifs for binding to EGR-1 consensus sequences in promoters of genes such as Insulin-like Growth Factor (IGF)-II and Platelet Derived Growth Factor (PDGF) – A chain[158-160]. The WT1 protein is normally expressed at its highest level during embryonic development. It shows low-level expression in adulthood, when it is confined to the nuclei of normal tissues such as thymus, spleen, haematopoietic progenitor cells, renal and gonadal cells[74,159].

Increased expression of WT1 messenger ribonucleic acid (mRNA) and/or protein has been demonstrated in various haematopoietic malignancies (AML, CML, ALL and MDS)[161-164]. Whilst data are conflicting regarding the prognostic implication of the level of *WT1* expression in AML patients at diagnosis[74,165-168], it is recognized that *WT1* is frequently overexpressed in their abnormal mononuclear cells. Studies have reported that *WT1* is highly expressed (as measured by quantitation of mRNA transcripts) in the AML blasts of at least 70% of newly diagnosed patients[163]. Expression of WT1 mRNA by AML blasts at diagnosis

has been shown to be 300-400 fold higher than that in normal bone marrow in one study[169]. More recently, data from a European LeukaemiaNet study using a standardized assay for *WT1* mRNA measurement, identified median expression of *WT1* mRNA by real-time quantitative polymerase chain reaction (RT-qPCR) in the peripheral blood (PB) and bone marrow (BM) of healthy controls to be 0.01 copies /10^4 ABL copies (range, 0.01-47.6) and 19.8 copies/10^4 ABL copies (range, 0-213) respectively in 204 healthy donor specimens. By contrast, analysis of 504 pre-treatment AML samples revealed median *WT1* expression in PB and BM to be 3107 (range 0-1.13x10^6) copies/10^4 ABL copies and 2505 (range 0-7.5x10^5) copies/10^4 ABL copies[67]. Far less frequently, mutations in *WT1* (usually insertions or deletions in exons 7 and 9) have been observed in paediatric and adult AML patients, particularly in normal karyotype AML or in association with *Flt-3* mutation[74]. In one study, 10% of cytogenetically normal AML patients harboured *WT1* mutations at diagnosis[170], however data are contradictory as to whether these mutations predict adverse outcomes[6].

The frequent overexpression of *WT1* gene products in AML patients renders it an attractive target for AML immunotherapy. However, *WT1* expression by healthy tissues is an important consideration, since the induction of WT1-specific immune responses could result in autoimmunity against renal glomerular podocytes or haematopoietic cells. During myeloid differentiation there is up-regulation of *WT1* expression by common myeloid, granulocyte-monocyte and megakaryocyte-erythroid progenitors (4%, 2% and 17% of cells respectively) but <1% of fully differentiated myeloid cells have detectable *WT1* expression[74,171]. *WT1* has been shown to be detectable in ~1% of CD34+ pluripotent haematopoietic progenitor cells (both in the committed (proliferating) CD38+ and uncommitted (quiescent) CD38- fractions) but not in long-term bone marrow haematopoietic stem cells[74]. Additionally, overexpression of *WT1* in CD34+ CD38- LSCs relative to healthy haematopoietic stem cells has been demonstrated[171,172]. Such endosteal BM resident CD34+ CD38- LSCs are non-cycling and therefore resistant to cytotoxic agents, but may be sensitive to targeting of WT1[60].

These findings collectively suggest that immunotherapeutic targeting of WT1 should predominantly affect malignant cells with abnormally high levels of WT1 expression, rather than normal tissues showing much

lower expression. Indeed, studies of the lytic function of WT1-specific CTLs have demonstrated selective elimination of leukaemic over healthy CD34+ cells. A high-avidity WT1-specific CTL line killed CD34+ cells isolated from CML patients but not healthy donor CD34+ cells. The difference in lysis was attributed to the much lower expression of *WT1* (determined by RT-PCR of extracted mRNA and Western Blotting) by healthy CD34+ cells in comparison with CD34+ cells from CML patients[173]. In addition, these researchers and a separate group showed no inhibition of colony formation by healthy donor bone marrow cells following co-culture with WT1-specific CTLs[173,174].

An additional feature rendering WT1 an attractive candidate for immunotherapeutic targeting in AML is its putative role in maintenance of the malignant phenotype. This has been proposed by the observation of reduced proliferation of leukaemic cells exposed to *WT1* antisense oligonucleotides and suggests that WT1 represents a persistent target in AML cells during the disease course[164]. Finally, *WT1* expression may serve as a marker of minimal residual disease (MRD) and early predictor of disease recurrence. Immunotherapeutic targeting of WT1 during a period of low disease burden, detected through monitoring of *WT1* mRNA in patients' PBMCs, could therefore represent an efficacious strategy[175].

1.6.2 Immunogenicity of WT1: vaccine-induced and spontaneous T-cell responses directed at WT1 epitopes in mice and humans

1.6.2.1 Induction of WT1-specific T-cell responses in murine models

Early studies exploited murine models to investigate putative immunogenic WT1 epitopes using peptide vaccinations. The DNA coding sequence of *WT1* shows a high level of conservation: approximately 81% sequence homology between murine and human cDNA with a 92% homology between the protein-encoding regions (Figure 1-4)[160,176]. Immunogenic epitopes from homologous sequences within both murine and human WT1 may therefore be selected for studies in murine models of WT1 peptide vaccination. WT1 also shows a similar pattern of expression in adult murine and human tissues, therefore autoimmune toxicity as a consequence of WT1 peptide vaccination in mice may predict toxicities in humans. Furthermore, successful induction of WT1-specific T-cell responses in mice suggests tolerance to

this self-antigen can be subverted, which is likely to be similarly required for effective induction of WT1-specific T-cell responses in humans.

Figure 1-4 Protein sequence alignment of murine and human WT1 protein

```
Human   2    QDPASTCVPEPASQHTLRSGPGCLQQPEQQGVRDPGGIWAKLGAAEASAERLQGRRSRGA  61
             Q+PASTCVPEPASQHTLR  PGC+QQPEQ G R P   WAK     +SAE  Q RRS
Mouse   7    QEPASTCVPEPASQHTLRREPGCVQQPEQPGDRGPRSAWAK-----SSAENPQDRRSGEP  61

Human   62   SGSEPQQMGSDVRDLNALLPAVPSLGGGGGCA-LPVSGAAQWAPVLDFAPPGASAYGSLG  120
             S SEP  MGSDVRDLNALLPAV SLGGGGG   LPVSGAAQWAPVLDFAPPGASAYGSLG
Mouse   62   SASEPHLMGSDVRDLNALLPAVSSLGGGGGGCGLPVSGAAQWAPVLDFAPPGASAYGSLG  121

Human   121  GPAPPPAPPPPPPPPPHSFIKQEPSWGGAEPHEEQCLSAFTVHFSGQFTGTAGACRYGPF  180
             GPAPPPAPPPPPP PPHSFIKQEPSWGGAEPHEEQCLSAFT+HFSGQFTGTAGACRYGPF
Mouse   122  GPAPPPAPPPPPP-PPHSFIKQEPSWGGAEPHEEQCLSAFTLHFSGQFTGTAGACRYGPF  180

Human   181  GPPPPSQASSGQARMFPNAPYLPSCLESQPAIRNQGYSTVTFDGTPSYGHTPSHHAAQFP  240
             GPPPPSQASSGQARMFPNAPYLPSCLESQP IRNQGYSTVTFDG PSYGHTPSHHAAQFP
Mouse   181  GPPPPSQASSGQARMFPNAPYLPSCLESQPTIRNQGYSTVTFDGAPSYGHTPSHHAAQFP  240

Human   241  NHSFKHEDPMGQQGSLGEQQYSVPPPVYGCHTPTDSCTGSQALLLRTPYSSDNLYQMTSQ  300
             NHSFKHEDPMGQQGSLGEQQYSVPPPVYGCHTPTDSCTGSQALLLRTPYSSDNLYQMTSQ
Mouse   241  NHSFKHEDPMGQQGSLGEQQYSVPPPVYGCHTPTDSCTGSQALLLRTPYSSDNLYQMTSQ  300

Human   301  LECMTWNQMNLGATLKGVAAGSSSSVKWTEGQSNHSTGYESDNHTTPILCGAQYRIHTHG  360
             LECMTWNQMNLGATLKG+AAGSSSSVKWTEGQSNH TGYES+NHT PILCGAQYRIHTHG
Mouse   301  LECMTWNQMNLGATLKGMAAGSSSSVKWTEGQSNHGTGYESENHTAPILCGAQYRIHTHG  360

Human   361  VFRGIQDVRRVPGVAPTLVRSASETSEKRPFMCAYPGCNKRYFKLSHLQMHSRKHTGEKP  420
             VFRGIQDVRRV GVAPTLVRSASETSEKRPFMCAYPGCNKRYFKLSHLQMHSRKHTGEKP
Mouse   361  VFRGIQDVRRVSGVAPTLVRSASETSEKRPFMCAYPGCNKRYFKLSHLQMHSRKHTGEKP  420

Human   421  YQCDFKDCERRFSRSDQLKRHQRRHTGVKPFQCKTCQRKFSRSDHLKTHTRTHTGKTSEK  480
             YQCDFKDCERRFSRSDQLKRHQRRHTGVKPFQCKTCQRKFSRSDHLKTHTRTHTGKTSEK
Mouse   421  YQCDFKDCERRFSRSDQLKRHQRRHTGVKPFQCKTCQRKFSRSDHLKTHTRTHTGKTSEK  480

Human   481  PFSCRWPSCQKKFARSDELVRHHNMHQRNMTKLQLAL  517
             PFSCRW SCQKKFARSDELVRHHNMHQRNMTKLQLAL
Mouse   481  PFSCRWHSCQKKFARSDELVRHHNMHQRNMTKLQLAL  517
```

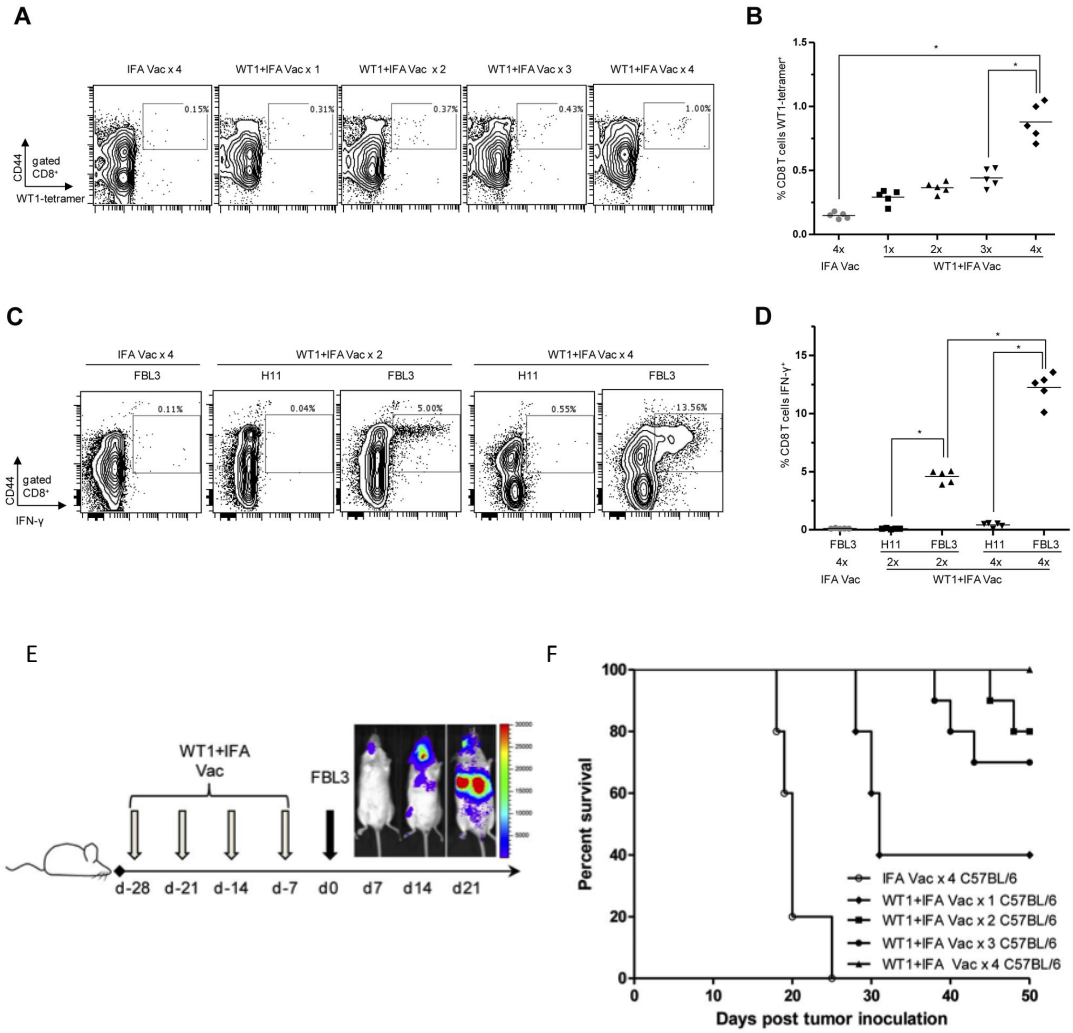
Alignment of murine and human amino acid sequences of WT1 (accession numbers NP_659032.3 and NP_077744.3 respectively) reveals 92% sequence homology. Human and murine sequences are aligned above; the intervening row outlines the homologous, missing and altered amino acids. Sequence alignment was performed using the ExPasy Bioinformatics Resource Portal (http://www.expasy.org)[177].

One of the earliest identified HLA-A*02:01 restricted WT1 epitopes, p126 (RMFPNAPYL, WT1-RMF), was initially shown to stimulate human HLA-A*02:01 specific CTLs *in vitro*[178] and subsequently to promote H-2D^{b}-restricted T-cell responses in C57BL/6 mice[179]. Sugiyama's group suggested comparable binding affinity of WT1-RMF to H-2D^{b} or HLA-A*02:01, a finding since supported by *in silico* HLA binding prediction software, such as SYFPEITHI (http://www.syfpeithi.de/index.html)[180] and the Immune Epitope Database (IEDB) (http://www.immuneepitope.org/)[181]. These programmes analyse proteins for the presence of sequence motifs (the set of anchor residues within the peptide which interact with the relevant binding

points within a particular HLA molecule) to enable the identification of putative immunogenic epitopes. In the case of SYFPEITHI, the top 2% of listed peptides in the output are predicted to have the highest binding affinity to a particular allele (generally scoring >20 on the binding index)[180]. Using the SYFPEITHI algorithm, binding scores for WT1-RMF to H-2D^{b} and HLA-A*02:01 are comparable, at 24 and 22 respectively[180].

Sugiyama's group demonstrated that C57BL/6 mice immunised with syngeneic, lipopolysaccharide-stimulated splenocytes loaded with WT1-RMF resulted in rejection of the WT1 over-expressing, syngeneic leukaemic cell line FBL-3, without toxicity against healthy tissue, such as glomerular epithelium[179]. Although other epitopes derived from WT1 have been explored in peptide vaccination studies using C57BL/6 mice[159], WT1-RMF remains the most extensively studied to date. Whilst induction of WT1-RMF specific CTL responses in mice following repeated vaccinations has been demonstrated, in general, vaccinations have been effective only in the prophylactic setting[179,182] (rejection of subsequent tumour challenge), with diminished therapeutic efficacy for established tumours[182-184]. This was most recently described by Kohrt et al., who vaccinated C57BL/6 mice up to 4 times at weekly intervals with WT1-RMF peptide in combination with Incomplete Freund's Adjuvant (IFA) and determined the frequency of WT1-RMF specific T-cells in peripheral blood after each vaccination using fluorescence-based tetramer analysis, Figure 1-5. After 4 rounds of vaccination, approximately 1-1.5% of all CD8+ T-cells were WT1-RMF specific. Following *in vitro* cultures, the percentage of IFNγ+ CD8+ memory T- cells responding specifically to WT1-expressing tumour cells was higher (at 10-15%), Figure 1-5. Mice immunized four times were able to subsequently reject FBL-3 cells resulting in superior survival compared with mice immunized with IFA alone or up to 3 total IFA/WT1-RMF doses[182].

Figure 1-5 WT1-RMF specific T-cell responses induced following repeated vaccination with WT1-RMF and Incomplete Freund's Adjuvant can result in subsequent rejection of WT1-expressing tumour but not established tumour



Mice were immunized with IFA alone, or with IFA & WT1-RMF, weekly for up to 4 vaccinations. Blood samples were collected at day 28 from all mice and analysed for WT1-RMF tetramer staining **(A-B)** and IFNγ production following *in vitro* culture (**C-D**). **A.** Percentages of CD44+ (effector memory) WT1-RMF tetramer+ CD8 T-cells at day 28 following 1-4 vaccinations (representative flow cytometry plots) **B.** Mean percentage of WT1-RMF-tetramer+ CD8+ T cells, n=5 per group. **C.** Peripheral blood lymphocytes were isolated from mice after 2 rounds of vaccination with WT1-RMF/IFA or 4 rounds of vaccination with either IFA alone or WT1-RMF&IFA. IFNγ production by CD8+ CD44+ T-cells was assessed after 24-hour co-culture *in vitro* with WT1+ FBL-3 or WT1- H11 tumour cell lines (representative flow cytometry plot from each group shown). **D** Mean percentage of IFNγ+ CD8+ T cells in each group (n=5 per group). **(E-F)** Mice were challenged with $1x10^5$ WT1+ FBL-3 cells 7 days after the last of up to 4 immunizations (**E**) and survival was analysed. **F** Survival duration after tumour challenge was significantly improved in mice immunized with WT1-RMF and IFA relative to mice receiving IFA alone ($p<0.01$ for all WT1-RMF immunized mice versus controls). Survival appeared to be prolonged with increasing number of vaccinations received (no statistical comparisons between the groups of WT1-RMF immunized mice provided in this publication).

Taken from Kohrt et al[182].

These data suggest that despite induction of low frequencies of WT1-specific T-cells following 4 vaccinations (determined by tetramer analyses), CTLs were of sufficient functional potency to subsequently reject tumour cells. However, therapeutic vaccinations with 4 doses of WT1-RMF and IFA, a day after inoculation with FBL-3, could not prevent death in any tumour-bearing mice[182]. Therefore in the context of active tumour growth, either the time taken to induce WT1-RMF specific T-cells or the suppressive tumour microenvironment prevented immune-mediated destruction of the tumour. Such data highlights the requirement for more effective vaccination strategies to target WT1-expressing tumours, described in later sections.

1.6.2.2 WT1-specific T-cell responses in healthy donors and patients

WT1-RMF/A*02:01-specific CTLs generated following prolonged cultures of healthy donor PBMCs have demonstrated significant lytic activity against A*02:01+ WT1-expressing leukaemic cell lines (up to 80% of targets at the highest effector: target ratios) and primary leukaemic cells (up to 60% of targets) *in vitro*[173]. A number of other Class I[174,185-187] and II[188-192] epitopes from WT1 have been identified through *in vitro* cultures of healthy donor and patient PBMCs with candidate peptides. WT1-specific CTLs generated from such cultures have shown specific cytotoxicity against WT1-expressing primary leukaemia cells or cell lines in a HLA-restricted manner, whilst sparing healthy CD34+ cells[173,186]. Adoptive transfer of donor-derived WT1 specific CTLs into non-obese diabetic, severe combined immunodeficient mice (NOD SCID) led to regression of WT1-expressing human leukaemia[173,186]. Detection of WT1-specific CTLs in the peripheral blood of patients with myeloid malignancies has provided evidence that WT1 is immunogenic in the setting of active disease[193,194]. In the context of allogeneic HSCT, detectable WT1-specific immune responses in donors and HSCT recipients have been associated with GvL activity[195-197].

Given this evidence supporting immunogenicity of selected WT1 epitopes, investigators have explored WT1 peptide vaccination as a means to induce WT1-specific cytotoxic T-cell responses against WT1-expressing tumours. A number of Phase I/II trials of WT1 peptide vaccination in patients with haematological malignancies have been carried out, a selection of which are described in more detail in Table 1-3. The majority of these studies (and also those in the setting of solid tumour) have used a single

Class I peptide derived from WT1 for vaccination. Patient cohorts have frequently comprised those with myeloid malignancies, specifically AML, MDS or CML. Subjects usually had detectable disease burden (morphological evidence of >5% leukaemic blasts on bone marrow examination or detectable WT1 mRNA transcripts in blood/bone marrow). Reductions in WT1 mRNA burden have followed WT1 peptide vaccination in case series or in clinical trials[198-202]. Similarly, withdrawal of vaccination was associated with recrudescence of WT1 mRNA transcripts in blood/bone marrow[175,202]. Immunological responses were detectable in some patients following vaccination, including increased frequencies of WT1 tetramer+ CD8+ T-cells and greater cytokine production (IFNγ or TNFα) by patient T-cells upon re-exposure to WT1 peptide *in vitro*[173,198-200]. In one study, delayed type hypersensitivity reactions were detected in a small number of patients who showed a sustained remission following vaccination[201]. Only one study, using a single Class I HLA-A*24:02 restricted peptide to vaccinate Japanese patients demonstrated a correlation between the induction of increased frequencies of WT1 tetramer+ T-cells following vaccination to reduction in tumour burden (percentage of BM leukaemic blasts and/or WT expression levels in BM/blood)[202]. The proportion of patients demonstrating immunological responses to WT1 peptide vaccination has varied between 44-86% in these studies. Overall, the magnitude of the induced WT1-specific T-cell responses has been small, with duration limited to weeks[175,198-200,202].

One approach to enhancing immune response induction to the well-studied epitope WT1-RMF that has been explored in pre-clinical and now clinical studies has been heteroclitic modification of this peptide to enhance binding to the HLA Class I binding groove. Scheinberg's group substituted the arginine at position one (a secondary anchor residue for MHC Class I binding) for a tyrosine residue (WT1-YMFPNAPYL, WT1-YMF)[203]. WT1-YMF was predicted *in silico* to have higher binding affinity for HLA-A*02:01 (SYFPEITHI score 24 versus 22 for the native WT1-RMF). Greater binding of WT1-YMF to the Class I peptide-binding groove may result in a higher density of WT1-YMF/A*02:01 complexes on APCs and more sustained presentation to CD8+ T-cells that cross-react with the native WT1-RMF/A*02:01 complex[203,204]. T-cell clones generated *in vitro* against WT1-YMF showed greater IFNγ production when re-exposed to the native or heteroclitic peptide in comparison to WT1-RMF-specific CTLs, but did not show superior lysis of primary WT1+ CML cells[203]. The group used WT1-YMF as part of their multi-epitope vaccine against WT1 (Table 1-3) and demonstrated cross-reactivity of CD8+ T-cells isolated from vaccinated patients against the native

peptide, by virtue of proliferative responses, cytokine production and lysis of WT1-expressing targets[201]. Overall, the use of heteroclitic peptides in cancer vaccines has shown variable efficacy, enhancing antigen-specific immune responses against some targets[205] or priming CTLs with inferior anti-tumour activity compared to CTLs stimulated with the native peptide[206]. As yet it is uncertain whether immunisations using WT1-YMF afford superior efficacy over WT1-RMF for induction of WT1-RMF-specific responses. This approach warrants further investigation in vaccination studies.

These early phase studies of WT1 peptide vaccination have primarily been designed to assess safety and feasibility of peptide vaccination targeting WT1. Minor toxicity has been reported despite evidence of WT1-specific immune responses being induced (Table 1-3). Indeed a handful of patients have been vaccinated over 100 times during an approximately 8 year treatment period with the only adverse events described being local injection site induration[175]. In summary, substantial *in vitro* and *in vivo* evidence from murine and human studies have suggested potential efficacy of peptide vaccination targeting WT1 as immunotherapy against myeloid leukaemia. Whilst clinical trial data supports induction of WT1-specific immune responses by vaccination, in some instances associated with a reduction in tumour burden, clear objective evidence of sustained and efficacious anti-leukaemic responses are lacking. In the next section, methods to enhance induction of WT1-specific immune responses are discussed.

Table 1-3 Clinical trials of WT1 peptide vaccination in patients with myeloid malignancies

§See Appendix A for classification of MDS subtypes

Study type and patient group, n	Vaccine components and mode of administration	Vaccination schedule	Clinical Results and Immunological Responses	Reference
Phase I HLA-A*24:02+ 26 cancer patients (13 AML/MDS with resistant disease)	$WT1_{235-243}$ (CMTWNQMNL) or heteroclitic CYTWNQMNL in Montanide ISA 51 at 0.3/1/3 mg of peptide per vaccination, i.d.	Vaccination fortnightly for 3 doses; further vaccinations permitted according to toxicity/response	10 AML patients completed ≥ 3 vaccinations with minimal toxicity(3 MDS patients did not complete vaccinstaions due to leucopenia). 9/13 (69%) patients had immunological responses to vaccination (>1.5 fold increase in frequency of WT1 tetramer+ T-cells post vaccination compared with baseline pre-vaccination frequency). Increased WT1 tetramer+ T-cells correlated with disease/clinical response to vaccination. 6 AML patients showed increase in WT1-specific IFNγ production by PBMCs post-vaccination.	202
Phase II A2+ Relapsed/refractory AML, MDS RAEB§ n=19	D1-4: GM-CSF s.c. D3: 200 μg WT1-RMF + 1 mg Keyhole Limpet Haemocyanin (KLH) i.d.	9 patients (cohort 1): 4 bi-weekly then monthly vaccinations 10 patients (cohort 2): continual bi-weekly vaccination. Vaccination until disease progression.	Median 11 doses delivered (range 4-27). Minimal toxicity. 8/18 (44%) patients showed evidence of an immunological response to vaccination.↑Median frequency of WT1 tetramer+ T-cells from baseline 0.12% to 0.25% (week 18). Proportion of patients with functional WT1-specific T-cell responses following *in vitro* culture rose during treatment: (baseline 11% of patients ➔39% at week 10 ➔ 50% at week 18). Disease stabilization (median 155 days) in 10 AML patients	198
Phase I+ II A*02:01+ AML in CR, CML, MDS-RA/RARS; n=8 Phase I, single vaccination n=7 Phase II repeated vaccination	200μg WT1-RMF and 500 μg VLQELNVTV (PR1-derived, A2-restricted peptide) single dose s.c. GM-CSF s.c. Administered in Montanide ISA 51 VG (water in oil emulsion)	Single dose administered in Phase I study 6 vaccinations every 2 weeks with booster at 12 weeks following last dose in Phase II	6 patients completed all 6 vaccinations (3 received the booster at 12 weeks). Minimal toxicity.↑WT1+ tetramers in 5 patients (62.5%) detected 1 week after 1st dose (median baseline 0.03% increased to median 0.29%). ↑Frequency of IFNγ+T-cells after dose 1 correlated with development of tetramer response.3 immunological responders also showed reduced peripheral blood WT1 mRNA transcripts. Immune responses were short-lived, not improved by repeated vaccination. Repeated vaccination was associated with deletion of high avidity T cells	199,200

Table 1.3 continued

Study type and patient group, n	Vaccine components and mode of administration	Vaccination schedule	Clinical Results and Immunological Responses	Reference
Pilot study Not selected for HLA type AML in CR but detectable WT1 mRNA transcripts in bone marrow samples, n=9	Multi-epitope peptide vaccine containing each 4 heteroclitic and long peptides: 1. YMFPNAPYL 2. RSDELVRHHNMHQRNMTKL 3. PGCNKRYFKLSHLQMHSRKHTG 4. SGQAYMFPNAPYLPSCLES 200μg per peptide admixed with Montanide ISA 51	6 doses (weeks 0,4,6,8,10, 12) Patients with stable disease, evidence of immune responses or fall in WT1 mRNA levels could receive up to 6 more vaccinations administered monthly	Minimum 3 and maximum 12 doses given to 9 patients. Minimal toxicity. 7 patients (78%) showed evidence of an immunological response to vaccination. Significant delayed-type hypersensitivity response in 3 patients of whom 2 remained in CR *In vitro* cultures to assess CD4+ and CD8+ T cell responses demonstrated CD4 proliferative responses to peptide(s), WT1-specific IFNγ production and WT1+ cell line lysis No clear treatment-associated impact upon WT1 mRNA levels. 4/9 patients relapsed within the follow-up period.	[201]
Phase I/II dose escalation study A*02:01+ Poor prognosis, allogeneic transplant ineligible AML, in CR or persistent disease n=8	Three dose levels assessed: 0.3/0.6/1.0 mg of each peptide per vaccination. 0.3/0.6/1.0 mg WT1-RMF + 0.3/0.6/1.0 mg PADRE peptide, and of 0.3/0.6/1.0 mg $WT1_{235-243}$ (CMTWNQMNL)+ 0.3/0.6/ 1.0 mg PADRE peptide mixed with Montanide ISA-51 to form 2 separate s.c. injections.	Vaccines were administered 3-weekly. 3 patients were vaccinated at the 0.3mg dose level, 3 at 0.6mg and 2 at 1mg. Up to 5 vaccination cycles allowed per patient.	7 patients received 4-5 cycles of vaccination (1 showed disease progression after 2 cycles). Minimal vaccine-related toxicity, local erythema and induration at vaccine sites noted. 6/7 (86%) patients showed an increase in the frequency of WT1 tetramer+ CD8+ T-cells at maximal post-vaccination response 1.5x above baseline (after *in vitro* culture with WT1 peptides median baseline frequency 0.1% increased to median 0.45% at maximal response). 6/7 patients showed ↑WT1-specific IFNγ production by T-cells after stimulation with all three peptides following vaccination. 2/5 patients showed reduction of WT1 mRNA transcripts and simultaneous increase in functional WT1-specific + T-	[207]

i.d., intra-dermal; s.c., subcutaneous

1.6.3 Limitations of vaccine approaches to date

While clinical studies indicate that WT1 peptide vaccination appears safe, it is difficult to gauge efficacy due to the inclusion of select patients, often with advanced stage disease, in uncontrolled Phase I-II trials. In such early phase studies, assessment of immune response induction has frequently been a secondary endpoint. There are likely to be inherent obstacles to the induction of immune responses against a self-antigen in cancer patients, namely the requirement to overcome peripheral tolerance mechanisms and the immune suppressive milieu within the tumour-bearing host. There are specific limitations to WT1 peptide vaccination approaches reported to date that should be considered when designing new vaccination strategies. Broadly, these can be divided into the choice of peptide(s) and adjuvants used for vaccination.

Choice of immunising WT1 peptides

The majority of early studies of WT1 peptide vaccination in both solid tumour and haematological malignancies have used a single HLA Class I peptide for vaccination[198-200,202,208]. Thus far, few studies have included Class II epitopes within WT1 peptide vaccines[201,209]. Incorporation of Class II epitopes is attractive, as activation of CD4+ helper T-cells may increase the likelihood of generating memory T-cell responses[210,211]. One reported trial of WT1 peptide vaccination not incorporating a helper epitope demonstrated short-lived WT1-specific CD8+ T-cell responses, suggesting poor memory T-cell response induction[200]. Furthermore, activated CD4+ T-cells may be capable of directly mediating tumour cytotoxicity[212]. AML and MDS patients with advanced phase disease have been shown to have significantly elevated WT1-specific Immunoglobulin-gamma (IgG) antibodies in their sera in comparison with early phase MDS patients and healthy volunteers[213]. This suggests a functional role for CD4+ helper T-cells in supporting WT1-specific adaptive immunity and taking these findings together, there is a clear rationale for the inclusion of epitopes to stimulate helper responses.

Synthetic long peptides (SLP) have been favoured over shorter Class II epitopes for inclusion in vaccines by some researchers[214]. Proposed benefits include a greater likelihood that CD4+ and CD8+ epitopes, both embedded within a carefully chosen SLP, will be processed by the same professional APC. This APC would

present appropriate epitopes simultaneously to CD4+ and CD8+ T-cells in close proximity. By contrast, use of shorter peptides could result in exogenous loading of minimal length determinants onto MHC molecules belonging to non-professional APCs, including B and T cells, which may travel to draining lymph nodes and drive transient or anergic responses in cognate T cells or perhaps even fratricide[214]. Scheinberg's group explored this approach by developing a multi-epitope WT1 vaccine including a Class I-binding nonamer, two Class II peptides and a SLP. The longer peptides were chosen for binding across a range of Class II molecules and had shown induction of both CD4+ and CD8+ T cell responses following *in vitro* cultures of healthy donor PBMCs[190]. This cocktail of WT1 peptides has demonstrated safety in two separate pilot studies of vaccination in AML[201] and lung cancer patients[209]. After *in vitro* culture of CD4+ and CD8+ T-cells isolated from vaccinated patients, IFNγ production, proliferative and lytic responses were observed upon exposure to targets presenting the immunising peptides[201,209].

A further advance would be the inclusion of multiple epitopes covering the whole target antigen sequence. By including several peptides spanning WT1, all known and as yet undiscovered immune epitopes are included within a single "off-the-shelf" vaccine product. Indeed, recent detailed *in vitro* studies by Doubrovina et al have identified 41 previously unreported immunogenic Class I and II epitopes within WT1, capable of binding across a broad range of HLA types[215]. The advantage of using an overlapping peptide pool rather than whole protein is that immunogenic epitopes would be more readily accessible for processing and presentation by professional APCs. Use of the whole protein for vaccination might increase the likelihood of generating a humoral rather than a cell-mediated response. Given that data is still accumulating regarding the identity of immunodominant epitopes within WT1 for a range of HLA types *in vivo*, vaccinating with a pool of overlapping WT1 peptides encompassing all potential epitopes is attractive. Overlapping peptide pools have been used in Phase II trials of therapeutic vaccination against E6 and E7 oncoproteins expressed by Human Papilloma Virus (HPV)-16–associated gynaecological malignancies[216,217]. Whilst it is acknowledged that the responses generated in these settings are against a foreign, tumour-specific, virus-derived antigen, nevertheless, therapeutic efficacy and detectable immune responses in vaccinated patients have been observed. Vaccinating with more than one immunogenic peptide may also prevent clonal deletion of T-cells following repeated vaccination against a single epitope[200].

Choice of adjuvant

A further explanation for the reported low magnitude and/or unsustained WT1-specific T-cell response to vaccination so far may be the choice of adjuvant used. Until recently, few adjuvants capable of promoting T-cell responses have been considered clinically safe enough to be used in trials. The majority of clinical studies of WT1 peptide vaccination have used adjuvants with well established safety records, such as Montanide (a derivative of incomplete Freund's adjuvant) and GM-CSF, Table 1-3[218]. Montanide formulations comprise mineral or non-mineral oil based adjuvants possessing surfactant properties that act as vehicles for vaccine delivery as well as inducing a local inflammatory reaction[219]. GM-CSF stimulates and matures DCs and it is suggested that this quality facilitates induction of T-cell responses following peptide vaccination against a self-antigen[220]. Such adjuvants have been widely used in clinical trials and shown to be safe, however their efficacy remains uncertain. In part, this is due to incomplete understanding of their modes of action. Some groups have raised concerns that vaccinations using Montanide-based emulsions containing Class I peptides were associated with induction of short-lived CD8+ T-cell activity and lack of memory response generation[200,221]. One explanation for this may be that slow, sustained release of the Class I peptide from the Montanide depot in the absence of helper or danger signals has a tolerising effect[221]. With regards to GM-CSF, two clinical trials of vaccination in melanoma, where patients were randomised to receive GM-CSF (or not) with their vaccine, demonstrated a statistically significant reduction in immunological responses for those patients who received GM-CSF[222,223]. It is possible that the local GM-CSF injection favoured activation of myeloid-derived suppressor cells that prevented an effective immune response from being generated. Whilst this observation may be vaccination dose or route dependent, it suggests that identification of more potent adjuvants for use in vaccinations is desirable[224].

Most clinical trials of peptide vaccination reported to date have employed the limited selection of adjuvants licensed for clinical use, generally adopted from the field of microbial vaccinology. Incomplete understanding of the mechanism of action of the earliest adjuvant formulations and concerns about the potential for induction of autoimmunity have contributed to delays in the development of the next

generation of compounds designed to promote cell-mediated immunity. Whilst the mode of action of some of the earliest identified adjuvants has only recently been appreciated, it is clear that many adjuvants exert their effects on B and T-cells via cells of the innate immune system. As such, there has been interest in exploiting vital players within the innate immune system, such as professional APCs that act as gatekeepers to effective T-cell priming and induction of adaptive immunity. Identification of compounds mimicking danger signals arising from infectious stimuli has driven the exploration of more potent mediators of cellular immunity, such as toll-like receptor (TLR) agonists, as vaccine adjuvants. The evidence and rationale to support this approach is discussed in the following section.

1.6.4 Novel adjuvants for induction of cell-mediated immunity

The importance of the innate immune system for priming adaptive immune responses has prompted the investigation of ligands known collectively as pathogen associated molecular patterns (PAMPs) as adjuvants. These ligands bind pattern recognition receptors (PRRs) on DCs to deliver a powerful danger signal that provokes their maturation into fully functional, professional APCs. Hijacking such ligands for use as adjuvants is one means to enhance response to vaccination. TLR agonists have been a particular focus of interest with selected agonists now licensed for clinical use as adjuvants whilst others remain under clinical trial investigation (Table 1-4).

TLRs are among the best-characterised PRRs (others include Nod-like receptors, retinoic-acid-inducible gene I (RIG-I)-like receptors, and C-type lectin receptors), having initially been identified in *Drosophila*. Thirteen TLRs have been identified, of which 10 are expressed in humans[225]. TLR function has been studied to greatest depth in the context of expression by DCs, but a range of cell types, including epithelial and endothelial cells, monocytes and lymphocytes express TLRs[225]. TLRs may be located at the cell-surface or internally located within endosomes and this spatial separation in part reflects the nature of the ligands binding these different receptors[226]. TLRs-1, -2, -4, -5, -6 and -11 are located at the cell-surface and primarily recognize lipid and/or protein-based components deriving from microbial walls[226]. One of the most well defined agonists binding to a cell-surface receptor is lipopolysaccharide (LPS), which binds to TLR-4 on macrophages. TLRs 3, -7, -8 and -9 are exclusively found within compartments such as the

endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes[226]. These receptors specifically recognize nucleic acids deriving from microbes that have been endocytosed into the cell[226].

Table 1-4 Licensed adjuvants in clinical use and adjuvants in experimental phase or late stage clinical development

Adjuvant	Major Immunostimulatory Component(s)	Innate Receptors or Pathway Activated	Principal Immune Responses Stimulated
Licensed Adjuvants			
Alum	aluminum salts	NLRP3 inflammasome (?)	Ab, Th2 (+ Th1 in humans)
MF59 and AS03	squalene-in-water emulsions	tissue inflammation (no receptors defined)	Ab, Th1 + Th2
AS04	MPL plus alum	TLR4 and inflammasome (?)	Ab, Th1
Adjuvants in Widespread Experimental Use or in Late Stage Clinical Development			
Poly-IC (also Poly-ICLC)	synthetic derivatives of dsRNA	TLR3, MDA5	Ab, Th1, $CD8^+$ T cells
MPL and formulations (AS01, AS02)	MPL and QS-21	TLR4 (MPL), ? (QS21)	Ab, Th1
Flagellin, flagellin-Ag fusion proteins	Flagellin from *S. typhimurium*	TLR5	Ab, Th1 + Th2
Imiquimods	imidazoquinoline derivatives	TLR7, TLR8 or both	Ab, Th1, $CD8^+$ T cells (when conjugated)
CpG oligodeoxynuceotides and formulations (IC31, QB10)	synthetic phophorothioate-linked DNA oligonucleotides with optimized CpG motifs	TLR9	Ab, Th1, $CD8^+$ T cells (when conjugated)
CAF01	trehalose dimycolate (cord factor)	Mincle	Ab, Th1, Th17
ISCOMS and ISCOMATRIX	saponins	mechanism undefined	Ab, Th1+ Th2, $CD8^+$ T cells
IFA (and Montanide formulations)	mineral or paraffin oil + surfactant	mechanism undefined	Ab, Th1 + Th2
CFA	IFA + peptidoglycan, trehalose dimycolate	NLR, inflammasome, Mincle, TLR?	Ab, Th1, Th17

The principal immune response stimulated is based on results from human and mouse studies, although it may be limited to one species in some cases. Where indicated, conjugation of TLR ligand to antigen is necessary to obtain significant $CD8^+$ T cell responses.

Ab, antibody; Th, T-helper 1/2/17. Taken from Coffman et al[227].

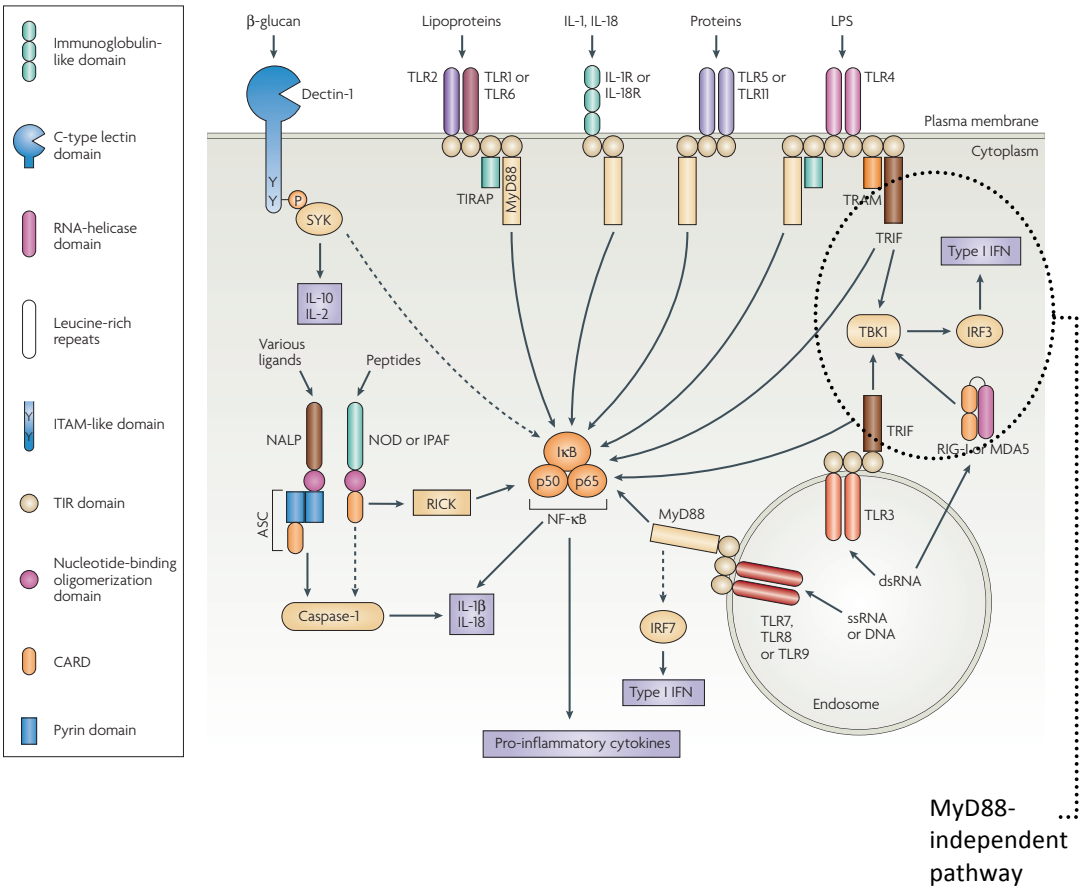
They are in this way segregated from the rest of the cell to avoid activation by the host's own nucleic acids. TLR3 was originally recognized to bind double-stranded RNA (dsRNA, typically derived from viruses) such as a synthetic analogue, polyinosinic-polycytidylic acid (poly I:C). TLR9 binds synthetic unmethylated 2'-deoxyribocytidine-phosphate-guanosine oligodeoxynucleotides (CpG ODN), based on DNA motifs present in microbes but infrequently found in mammalian cells[226]. Binding of CpG ODN to TLR9 directly activates DCs, macrophages and B-cells and is involved in skewing towards a T-helper (Th) 1-type of immune response[228], a function that may be appropriated for induction of cell-mediated response against tumours, described later.

TLR ligation leads to the recruitment and activation of a number of signalling proteins, including Mitogen activated protein (MAP) and Phosphoinositide-3 (PI3) kinases. Two major signalling pathways are activated following TLR ligation: a myeloid differentiation primary response gene 88 (MyD88)-dependent

and a MyD88-independent pathway (Figure 1-6)[229]. Signalling via these pathways results in distinctive biological consequences. TLRs 3 and 4 signal via the Toll/IL-1R (TIR)-domain-containing adaptor protein inducing IFNβ (TRIF), resulting in activation of the transcription factors Interferon regulatory factor (IRF) 3 Nuclear Factor kappa B and (NFκB). This leads to secretion of type I IFNs as well as inflammatory cytokines, whereas other TLRs (1,2,5 and 6) induce predominantly inflammatory cytokines via the MyD88-dependent pathway and activation of NFκB[226]. Intracellular TLRs 7 and 9 are primarily expressed by plasmacytoid DCs in humans, responding to microbial nucleic acids. These receptors signal via a MyD88-dependent pathway to trigger inflammatory cytokine and Type I IFN release, vital for the destruction of targets such as viruses[226]. The different cellular locations and signalling pathways used by TLRs direct the nature of the immune response which will be orchestrated by DCs, monocytes and other innate immune cells[226].

Ligation of TLRs on immature DCs provides a powerful stimulus for maturation into professional APCs. Binding of poly I:C to TLR3 within DCs results in enhanced MHC molecule expression, production of pro-inflammatory cytokines such as IL-12 and Type I IFN and enhanced cross-priming. Cross-priming allows induction of a cytotoxic CD8+ T-cell response to epitopes from endocytosed microbes, predominantly occurring via a retrograde pathway that delivers endocytosed pathogens to the cytosol. Following proteasomal degradation, peptides are delivered by transporter associated with antigen processing (TAP) transporters to MHC Class I molecules in the ER[230]. This is an important mechanism in the context of immunisation, as long peptides delivered into the dermal tissues must be endocytosed by DCs and transferred to the Class I pathway if presentation to CD8+ T-cells is to be possible. TLR9 ligation on murine plasmacytoid DCs has also been associated with enhanced cross-priming[231] and there is accumulating evidence that human DC subsets show increased cross-priming following exposure to selected TLR agonists[232,233]. Additionally, selected TLR agonists may stimulate innate/bystander cells (pDCs, non-haematopoietic cells) to secrete pro-inflammatory cytokines that act indirectly to promote myeloid DC antigen presentation to CD8+ T-cells[227].

Figure 1-6 Schematic representation of selected TLR signalling pathways.



Diagrammatic representation of signalling pathways used by selected TLRs. Cell-surface TLRs such as TLRs 1, 2, 4-6 signal via a MyD88 dependent pathway, as do the endosomally located TLRs 7-9. TLRs 3-4 utilise a MyD88 independent pathway that requires Toll/IL-1R (TIR)-domain-containing adaptor protein inducing IFNβ (TRIF). Signalling via TLRs 3-4 and 7-9 leads to increased production of Type I IFN by the DC as well as secretion of pro-inflammatory cytokines. Signalling via the other receptors predominantly results in secretion of pro-inflammatory cytokines such as IL-12, IL-10, IL-6, IL-1β and TNF.

Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD (caspase-recruitment domain); ds, double-stranded; IFN, interferon; IκB, inhibitor of NF-κB; IL, interleukin; IPAF, ICE-protease-activating factor; IRF, IFN-regulatory factor; LPS, lipopolysaccharide; MDA5, melanoma-differentiation-associated gene 5; MyD88, myeloid differentiation primary-response gene 88; NALP, NACHT-, LRR- and pyrin-domain-containing protein; NOD, nucleotide-binding oligomerization domain; RICK, receptor-interacting serine/threonine kinase; RIG-I, retinoic-acid-inducible gene I; ss, single-stranded; TBK1, TANK-binding kinase 1; TIRAP, Toll/IL-1R (TIR)-domain-containing adaptor protein; TRAM, TRIF-related adaptor molecule; SYK, spleen tyrosine kinase.

Figure adapted from Trinchieri et al[229].

Ligation of TLRs on tissue-resident DCs in the presence of an inflammatory milieu and signals e.g. from phagocytic receptors, induce their maturation and activation. As well as enhanced antigen processing and presentation, activated DCs up-regulate the chemokine receptor CCR7, enabling migration to the T-cell rich areas of lymphoid tissue. Local chemokines induce DCs to express high levels of co-stimulatory molecules (such as CD80, CD86 and CD40) and adhesion molecules such as Intercellular Adhesion Molecule-1 (ICAM-1) to increase the likelihood of successful interactions with naïve CD8+ and CD4+ T-cells. Secretion of IL-12 by activated DCs delivers the powerful "third signal" required to drive a Th1 immune response involving activated cytotoxic T-cells[234].

Researchers have demonstrated that a single TLR agonist or inflammatory cytokine alone is usually insufficient to optimally induce IL-12 production (specifically IL-12p70, the biologically active form of IL-12) by DCs. This suggests that similarly to T-cells, DCs also require two signals for high level secretion of this potent cytokine capable of inducing cytotoxic T-cell responses[229,234]. Indeed, ligation of certain combinations of TLRs has a cooperative effect on cytokine production (including TNF, IL-1β, IL-6, IL-10, IL-12 and IL-23) by DCs[229]. In one study of mouse macrophages, the use of two TLR agonists (poly I:C, targeting TLR-3 and CpG ODN, targeting TLR-9) resulted in supra-additive production of TNF, IL-6 and IL-12p40[235]. One explanation underlying this synergism is the activation of both the MyD88 dependent and independent signalling pathways using this combination of TLR agonists. Combined triggering of selected TLRs on mouse and human DCs also increases IL-12p70, promoting Th1 responses, enhanced IFNγ production and cytolytic activity in CD8+ T cells and NK cells[236,237].

These observations have led to the exploration of TLR agonists for use as adjuvants in vaccinations against both microbial targets and also tumours. A number of Phase I-III clinical trials to evaluate individual or combined TLR agonists as adjuvants in therapeutic vaccines for solid organ and haematological malignancies are ongoing. A Phase III trial of a Glaxo-Smith-Kline developed vaccine targeting melanoma-associated antigen (MAGE), in combination with TLR 4 and 9 agonists in melanoma patients, is due to report in the near future. It is hoped that regulatory approval for clinical use for many more of these adjuvants will be forthcoming as a result of such studies. A specific focus of our research group has been

to determine whether combining TLR agonists with other pro-inflammatory signals could further potentiate induction of antigen-specific T-cell responses. Our findings led to the development of a multi-adjuvant system known as CASAC – Combined Adjuvants for Synergistic Activation of Cellular immunity. A description of the relevant previously described studies of CASAC follows, as investigation of CASAC in combination with WT1 peptide vaccination forms a major portion of this thesis.

1.6.5 Combined Adjuvants for Synergistic Activation of Cellular Immunity (CASAC)

Work from our group investigated the potential for using novel adjuvant combinations in peptide vaccinations targeting both foreign and self-antigens[238]. In these studies C57BL/6 mice underwent two rounds of intra-dermal vaccination using an oil-in-water emulsion admixed in a 50:50 ratio with vaccine components. The vaccine consisted of combinations of up to two TLR agonists, IFNγ and agonist anti-CD40 antibody or a Class II helper peptide along with the target Class I peptide. Exogenous IFNγ (derived from activated NK and T-cells *in vivo*) or ligation of CD40 on DCs (via agonistic anti-CD40 antibody *in vitro* or activated T-cells expressing CD40L *in vivo*) have both been shown to combine with TLR ligation to increase IL-12p70 production, providing a rationale for their inclusion in the vaccine[239,240]. Fluorescent pentamer staining of CD8+ T-cells, IFNγ production and *in vivo* cytotoxic lysis assay were used to assess magnitude and functional efficacy of antigen-specific responses[238].

Following vaccinations against the chicken ovalbumin-derived OVA-SIINFEKL$_{257-264}$ (OVA-SIINF) peptide, fluorescently labelled H-2K^{b}-OVA-SIINF pentamers were used to identify cognate CD8+ T-cells. Massive clonal expansion of CD8+ T-cells specific for OVA-SIINF, up to 80% of all CD8+ T-cells, was observed following 2 vaccinations using the most potent combination of components: 2 TLR agonists (CpG ODN (TLR9 agonist) and monophosphoryl lipid A, MPL (TLR4 agonist)), agonist anti-CD40 antibody, IFNγ and 100 μg OVA-SIINF peptide, in emulsion. This combination of components was termed CASAC, or Combined Adjuvants for Synergistic Activation of Cellular Immunity[238].

These expansions of CD8+ OVA-SIINF-specific T-cells were capable of highly significant *in vivo* lytic activity, as measured by specific lysis of 90-100% of syngeneic splenocytes loaded with the immunising peptide. A functional, long-lived memory response was also observed using OVA-SIINF-specific pentamer analyses following re-challenge with OVA-SIINF peptide alone at >100 days post initial vaccination[238].

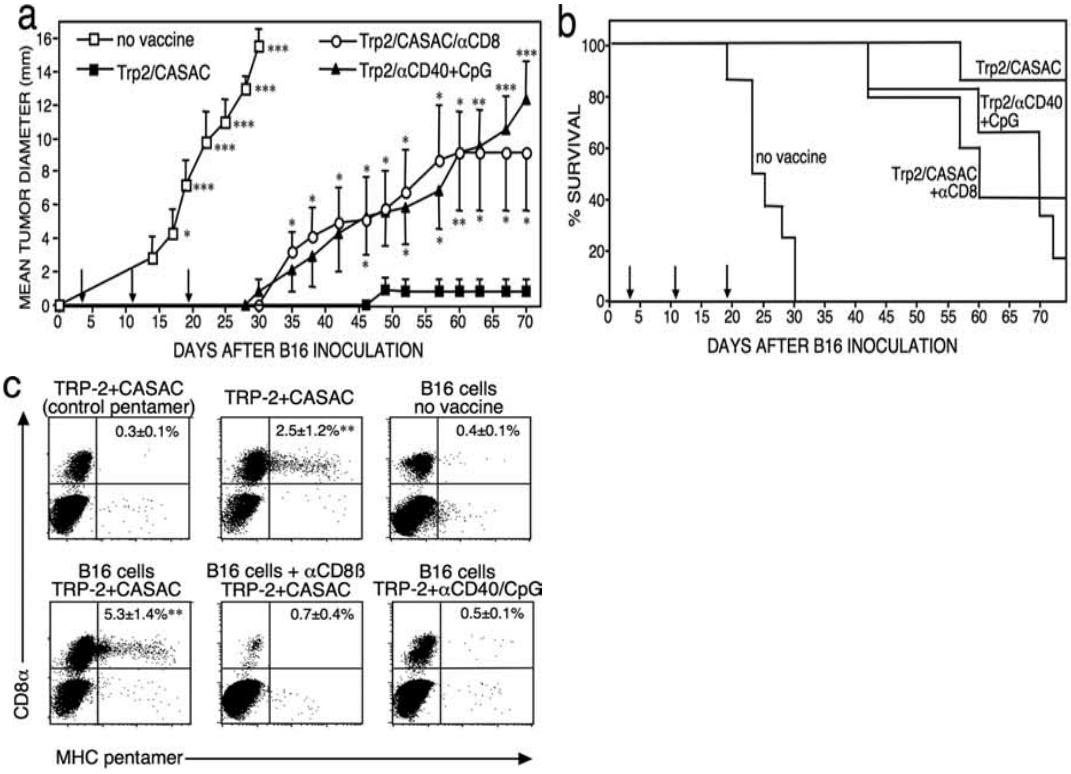
Different elements of the vaccination were assessed and optimised in this study. The major findings were as follows:

1. Relevant (derived from the same target protein as the Class I peptide) or irrelevant (unrelated) Class II helper peptides could substitute for agonist anti-CD40 antibody in CASAC to induce effective antigen-specific CD8+ T-cell responses. This suggests that ligation of CD40 on DCs by CD40L on activated helper T-cells may be one of the functions of these cells in driving cytotoxic T-cell responses, as has been reported previously[241,242].
2. Use of whole ovalbumin (OVA) protein for immunisation resulted in OVA-SIINF-specific CD8+ T-cell responses. This suggests that processing and cross-presentation of this class I epitope from OVA by CASAC-activated DCs occurred.
3. Presence of two TLR agonists in the vaccine was critical to efficacy. Some combinations of TLR agonists were more potent than others, e.g. CpG ODN and poly I:C were a more effective combination than CpG ODN plus Pam_3CSK_4 (TLR 1/2 agonist) and similar in potency to CpG ODN and MPL.
4. Analysis of cytokine production by CD4+ T cells isolated from draining lymph nodes suggested that Th1-type immune responses had been induced.
5. CASAC showed superior efficacy in generating OVA-SIINF-specific CD8+ T-cell responses over the most commonly used experimental adjuvant, Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA)[238].

Another key finding was that CD8+ T-cell responses could also be generated against a self-peptide derived from the melanoma associated antigen tyrosinase-related peptide 2 (TRP-2), $SVYDFFVWL_{180-188}$. A dose dependent increase in vaccination efficacy (frequency of TRP-2-specific CD8+ T-cells and antigen-specific *in vivo* lysis) was observed with up to 400μg of Class I peptide per vaccination. Furthermore, C57BL/6 mice inoculated with the TRP-2-expressing B16 syngeneic melanoma cell line showed significantly superior

survival following therapeutic vaccination with CASAC and TRP2 in comparison with control, non-immunised mice (Figure 1-7). This survival advantage was decreased by pre-treatment with depleting anti-CD8β antibodies, suggesting an important protective role for CD8+ T-cells in the anti-tumour response. Importantly, while there was some demonstrable evidence of autoimmunity in these animals (development of white hairs at the vaccination sites) there was no evidence of epitope spreading or destructive autoimmunity, despite the potency of the anti-B16 tumour response[238].

Figure 1-7 Vaccinations combining CASAC with a self-peptide can improve survival from melanoma in a mouse model



C57BL/6 mice (n=5-7 per group) were inoculated subcutaneously with 10^5 B16 melanoma cells per mouse. Mice were vaccinated at days 3, 11 and 19 with PBS only, or the class I TRP-2 peptide along with CASAC (comprising poly I:C, CpG ODN, IFNγ and anti-CD40 antibody) or agonist anti-CD40 antibody and CpG ODN . One group of CASAC immunised mice also received a depleting anti-CD8 antibody. (a,b). Reduced tumour diameter and improved survival were observed for mice immunised with TRP-2/CASAC, the effects of which were diminished by pre-treatment with depleting anti-CD8 antibody.
(c) The highest percentages of TRP-2 specific CD8+ T-cells by pentamer studies are detected in mice exposed to TRP-2 and CASAC. TRP-2 specific T-cells are detected even in tumour-bearing mice after immunisation. The frequency of antigen-specific T-cells is reduced by depletion of CD8β+cells or use of only one TLR agonist (CpG ODN) along with agonist anti-CD40 antibody.

Taken from Wells et al[238].

This work extended the findings of others who had explored combinations of a single TLR agonist with anti-CD40 antibody for vaccinations and demonstrated significant expansions of antigen-specific CD8+ T-cells showing lytic activity[243,244]. Direct comparisons between groups are difficult due to variations in methodology, for example the use of whole antigen versus peptide, and the number and interval of vaccinations. However, within the data presented by Wells, when a single TLR agonist and anti-CD40 antibody were used for vaccination, this appeared to be less efficient than CASAC in vaccinations against both foreign and self peptides. This suggests that the combination of multiple different signals may still have an additive effect upon immune response generation.

Previous work from our group has demonstrated the ability to use a novel adjuvant combination (CASAC) to induce highly potent immune responses against a xenoantigen. Additionally, effective anti-tumour responses were demonstrated following CASAC/self-peptide vaccination targeting a melanoma-associated tumour antigen. Given the apparent low magnitude and efficacy of immune responses activated against WT1 peptides using common adjuvants such as Montanide or GM-CSF, the potential efficacy of CASAC warrants exploration in the context of WT1 peptide vaccination and forms part of the data presented in this thesis.

1.7 Whole tumour cell vaccination using CD80/IL-2 gene modified blasts for immunotherapy of AML

Peptide vaccination targeting a single or few LAAs is an attractive immunotherapeutic strategy where immunogenic LAA epitopes have been identified. Furthermore, synthesis of peptides is a straightforward procedure culminating in the production of an “off the shelf” vaccine. There are however limitations to targeting a single antigen, namely that this may lead to immune escape by the tumour down regulating expression of the LAA. Furthermore, although certain LAAs are immunogenic and highly expressed by the tumour, there remains a lack of understanding regarding which LAA(s) is/are the crucial target(s) for therapy. It is likely that there are other as yet unidentified LAAs that are immunogenic.

One means to circumvent these issues is to use whole tumour (cell line or primary leukaemia cells) for vaccination. This has the benefit of including all potential target LAAs (known and unknown) and where primary leukaemic cells are used, results in the production of a vaccine that is tailored to the individual patient. Leukaemic blasts are particularly suitable as substrates for use in whole tumour cell vaccination. They are easily collected from peripheral blood and/or bone marrow for vaccine production. As described in section 1, AML cells may stimulate cognate T-cells and NK-cells through expression of MHC molecules and ligands for NK recognition, as well as adhesion molecules permitting interaction with immune cells. Due to their lineage origins, AML blasts come into close contact with cells of the innate and adaptive immune systems within haematopoietic tissues. Despite such qualities, they frequently fail to effectively activate responses in T- and NK-cells, due, for example, to secretion of soluble factors that adversely modulate the immune environment[101,102] or induction of anergy through insufficient expression of co-stimulatory molecules[99,245]. Therefore, genetic modification of AML cells to directly increase their antigen presentation function may increase their immunogenicity at vaccination. Immune responses may also be induced indirectly, as irradiated AML cells administered at vaccination will ultimately be engulfed by DCs, allowing presentation of tumour-derived antigens to lymphoid cells in draining lymph nodes.

Genetic modifications to induce expression of pro-inflammatory cytokines (such as GM-CSF[122,246,247], IL-4[248], IL-12[249] and TNFα[250]), some of which may aid maturation of AML cells into APCs, have been explored by investigators. Leukaemic cells modified to express GM-CSF have been evaluated in early phase clinical trials of whole cell vaccination. Researchers have carried out genetic modification of the CML cell line K562 to express GM-CSF, for use as a whole cell vaccine known as GVAX[251]. In a pilot study of GVAX vaccination in 19 CML patients not fully responsive to the tyrosine kinase inihibitor Imatinib, 13 patients experienced a reduction in disease burden. However, clear evidence of vaccine-specific immune responses was not described[247]. A mixture of GVAX and irradiated autologous leukaemia cells formed a patient-specific vaccine that was administered to AML patients undergoing autologous HSCT in a single arm, Phase II study. An immune readout in this study was development of a delayed type hypersensitivity (DTH) reaction following re-challenge with vaccine components (defined in general as persisting induration >10mm diameter after 48-72 hours and frequently associated with histologic characteristics including T-cell infiltration)[252]. A significant increase in 3y-relapse-free survival (100% vs. 48%, *p*=0.029)

was reported for 7/19 vaccine recipients who developed a DTH response versus those who did not[122]. Primary leukaemia cells modified by an adenoviral vector to express GM-CSF have been used to vaccinate AML and MDS patients at high risk of relapse following non-myeloablative allogeneic HSCT in a Phase I Clinical trial. DTH reactions were observed in 7 of 8 subjects who received 5 vaccinations, all of whom had experienced durable remissions at the time of reporting[246].

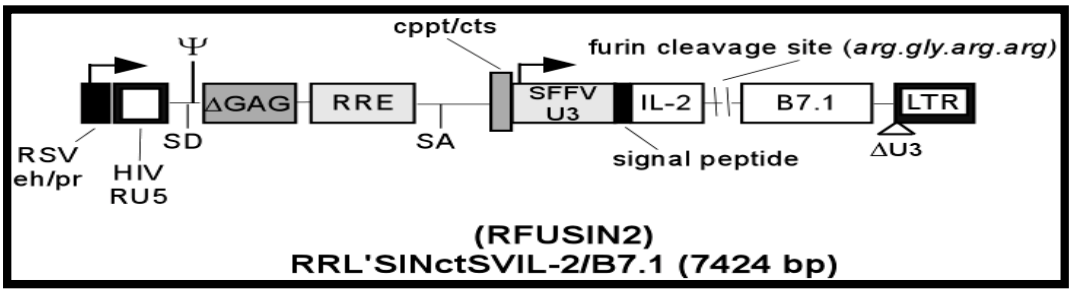
AML blasts express some immune stimulatory molecules, such as CD86, but reduced or absent expression of others, such as CD80[99]. In early studies of genetic modification of primary AML blasts, investigators developed vectors to introduce CD80 alone into AML cells in order to demonstrate induction of tumour specific immune responses *in vitro* and in animal models. CD80-modified AML blasts induced vigorous proliferative allogeneic CD4+ and CD8+ T-cell responses that were not seen in control cultures[253,254]. Mice immunised with AML cells modified to express CD80 subsequently rejected challenge with wild type, unmodified AML, which was not observed in unimmunised mice[255].

Latterly, researchers demonstrated that anti-leukaemic responses could be further enhanced by combined genetic modification of AML cells to express CD80 and pro-inflammatory cytokines. In murine models, superior survival and subsequent rejection of wild type tumour challenge was observed in mice immunised with leukaemic cells modified to express CD80 and GM-CSF compared with recipients of vaccine expressing either molecule alone[256]. Given demonstration of therapeutic efficacy in the *in vivo* setting, additional focus was placed on designing vectors likely to offer the greatest safety when administered to patients. Stripecke's group combined introduction of CD80 and GM-CSF expression into primary AML blasts using an HIV-based, self-inactivating lentiviral vector capable of effective induction of autologous and allogeneic T-cell responses[257].

Work in our laboratory over the last 20 years has focused on the generation of lentiviral-modified AML blasts (LV_AML) to induce expression of CD80 and secretion of IL-2[258]. IL-2 was chosen for inclusion in the vector given its ability to reverse T-cell clonal anergy and induce T and NK cell proliferation[259]. Lentiviral vectors show a number of advantages over the adenoviral and retroviral vectors for this application.

Firstly, they are able to efficiently transduce cells dividing even at a slow rate, such as AML cells in culture, contrasting with the other vector types. Secondly, lentiviral vectors can be pseudotyped by using vesicular stomatitis virus glycoprotein (VSV-G) envelope gene instead of the Human Immunodeficiency Virus (HIV)-1 envelope gene, permitting infection of a range of tissue types[260]. The lentiviral vector designed by Chan et al (RFUSIN2, Figure 1-8) incorporates a number of safety features, including the elimination of any viral gene-encoded proteins within a 4-plasmid system, suggesting a very low risk of generating replication-competent lentivirus[258]. Deletion in the 3' long terminal repeat (LTR) U3 region of the vector removes all potential HIV-1 LTR promoter activity such that the vector is self-inactivating upon genomic integration[258].

Figure 1-8 RFUSIN2 lentiviral construct



Taken from the Investigational Medicinal Product Dossier (IMPD) for the RFUSIN2-AML1 trial (EudraCT Number 2005-000806-29). See the main Abbreviations section for full explanation of terms.

Unpublished data from our department has demonstrated that survival of C3H mice inoculated with 32D leukaemia cells could be prolonged significantly following vaccination with irradiated RFUSIN2-transduced, CD80/IL-2 expressing 32D cells, administered between 10-20 days after tumour inoculation. Survival was greatest (80% at 100 days) for mice vaccinated with CD80/IL-2 modified cells, followed by those immunised with singly CD80 or IL-2 –modified blasts, with no mice surviving after vaccination with irradiated unmodified cells. These data confirmed the therapeutic potential of immunisation using CD80/IL-2 modified cancer cells and underlined the possible added benefit of combining both CD80 and IL-2 genes in the vector.

RFUSIN2 has been shown to efficiently transduce primary AML cells from a range of morphological and karyotypic subtypes. In published data, following RFUSIN2 transduction, between 41-98% of primary AML blasts expressed CD80, with IL-2 secretion ranging from 2-17ng/10^6 cells in 24 hours. As a comparator, transduction of AML cell lines resulted in 97-99% of cells expressing CD80 and IL-2 production was

between 9-11ng/10^6 cells in 24 hours[258]. Transduced primary AML cells (LV_AML) or unmodified AML cells isolated from patients at AML presentation were co-cultured *in vitro* with their matched remission PBMCs post-HSCT. Following co-culture, PBMCs were re-challenged with autologous wild-type AML or remission BM cells. PBMCs from a patient that had been co-cultured with LV_AML showed greater proliferative responses upon re-challenge with their wild type AML than PBMCs derived from cultures with unmodified blasts. Those PBMCs that had been previously stimulated by LV_AML showed less proliferation on exposure to healthy BM cells, suggesting leukaemia specificity of immune responses[258].

More detailed studies of patient T- and NK-cell responses following *in vitro* exposure to LV_AML cells have subsequently been published. PBMCs from patients, or T-cells from healthy donors were co-cultured with unrelated patients' CD80/IL-2 modified or wild-type AML blasts ("allogeneic" co-cultures). Upon subsequent challenge with the same blasts (wild-type), superior lysis was observed where PBMCs or T-cells had been previously co-cultured with LV_AML[120,121]. An additive effect on lytic response was observed where the vector conferred both CD80 and IL-2 expression, relative to vectors containing either component alone[120,121]. T-cells isolated post co-culture with LV_AML were shown to specifically secrete IFNγ following challenge with primary AML blasts rather than normal CD14+ bone marrow cells[120]. This is suggestive of some specificity of the T-cell response against abnormal, malignant cells rather than healthy bone marrow cells. Not only allogeneic/healthy donor T- and NK-cells but also patient-derived, autologous, remission T- and NK-cells could be stimulated by LV_AML to show anti-leukaemic cytotoxicity[120,121]. One important concern had been that IL-2 secretion by LV_AML cells might induce expansion of Tregs with potentially immunosuppressive effects on effector T-cell function. Studies carried out to specifically address this important issue demonstrated that despite evidence of Tregs in the co-cultures, subsequent lysis of primary AML blasts was preserved. There was no evidence of consistent expansion of Tregs following co-culture of PBMCs from healthy donors or patients with LV_AML[261]. Significantly, these studies confirmed that primary leukaemic blasts retained sensitivity to lysis *in vitro* by LV_AML stimulated donor-derived T- and NK-cells. This suggests that LV_AML can stimulate leukaemia-specific cytotoxic responses in lymphocytes post-allogeneic HSCT. Given that current reports describe low rates of response to tDLI, our pre-clinical data support use of LV_AML blasts as a whole cell vaccine to combine with freshly infused donor lymphocytes in order boost the induction of GvL activity.

These data allowed the approval of a Phase I trial of therapy of CD80/IL-2 modified AML blasts in patients with relapsed AML post allogeneic HSCT (RFUSIN2-AML1 vaccine trial, EudraCT Number 2005-000806-29). Blasts collected at the time of disease relapse are cryopreserved under Good Manufacturing Practice (GMP) conditions. Following salvage chemotherapy, patients showing morphological remission of AML (<5% BM blasts) and meeting eligibility criteria are allocated to treatment either with a patient-specific vaccine produced by RFUSIN2 transduction of the patient's stored AML blasts and escalating doses of DLI or DLI alone. The treatment arms are schematically presented in Figure 1-9, taken from the trial protocol (Appendix C).

Figure 1-9 Schematic depiction of treatment schedules in each arm of the RFUSIN2-AML1 trial

The primary endpoint of the study is to demonstrate safety of vaccination. One aspect of the safety investigations is to exclude any generation of replication competent lentivirus (RCL) by screening for the presence of HIV following vaccination. The specific vector-related safety questions that will be addressed are as follows:

1. Exclusion of viral genetic integration into the host genome. PCR studies will ascertain whether patient PBMCs show evidence of integrated viral genes in genomic DNA and RNA
2. Exclusion of RCL generation. Measurement of serum p24 will determine whether a functional lentivirus has been generated.
3. Exclusion of systemically elevated levels of IL-2. Detection of elevated systemic IL-2 in patients would be an unexpected and unwanted side effect of vaccination.

Patients will also be evaluated for evidence of exaggerated, systemic, immune stimulatory consequences of CD80 and IL-2 expression by LV_AML blasts, including:

1. Graft-versus-Host Disease. The use of donor lymphocyte infusions is associated with a risk of triggering either acute or chronic GvHD[262]. Given that CD80/IL-2 modified AML blasts are able to activate donor T-cells *in vitro*, it is possible that the risk of GvHD inherent to administration of DLI may be further exacerbated by the use of an immunostimulatory vaccine that may bear disparate mHags. Not only could this result in exacerbation of GvHD (and GvL) but this may also cause immune responses to be directed against normal haematopoietic precursors bearing such antigens.
2. Capillary Leak Syndrome and Cytokine Storm. The effective use of systemic IL-2 at high doses is often hampered by limited tolerability of this therapy[263]. Whilst some of the side effects result in poor tolerability of the treatment (e.g. due to fevers, chills, rigors), others are potentially life-threatening[263]. One such severe toxicity is capillary leak syndrome, where extravasation of vascular fluids and proteins into extravascular tissues results in fluid shift and accumulation of oedema[263]. This results in hypotension and poor organ perfusion and subsequent organ damage. The mechanisms underlying this include increased expression of endothelial cell adhesion molecules due to exposure to IL-2 and increased trafficking of recruited neutrophils, monocytes and lymphocytes across the endothelial wall. Damage to endothelial cell walls by activated

transmigrating cells may result in increased vascular permeability and fluid leak[264]. With the intra-dermal introduction of a very low dose of IL-2 associated with the CD80/IL-2 modified AML blasts (predicted to be <2.5 μg/24 hours), the likelihood of such an occurrence is small but will be monitored in this study.

The use of systemic immune activating agents has the potential to trigger an overwhelming immune response resulting in massive cytokine release with sequelae including multi-organ damage. This was observed in stark reality in the clinical trial of a super agonist anti-CD28 monoclonal antibody that resulted in massive stimulation of T-cells in the absence of TCR ligation[265]. Such a response would seem unlikely to occur in the context of the current clinical trial, as the CD80 protein should remain fixed to the cell-surface of the irradiated AML blast rather than freely circulate. The very low levels of IL-2 produced by the vaccine would suggest a low risk of hyperstimulation of the immune system due to IL-2, but this remains a focus of safety monitoring.

3. Off-target "autoimmune" reactivity. *In vitro* studies have suggested that there is little responsiveness of LV_AML primed T-cells against healthy bone marrow-derived CD14+ cells[120]. However, as AML blasts will share antigens with healthy tissues, induction of autoreactive T-cell responses remains a possibility. Furthermore, target cell destruction and local inflammation could result in other tissue-derived antigens being presented, resulting in off-target alloreactivity manifesting as autoimmune disease. During the course of treatment, clinical evaluation and autoantibody screening will be performed to identify evidence of autoimmune reactivity.

The above complications form the basis for characterising dose-limiting toxicity (DLT), which is defined by the development of vaccine-related Grade 3 or higher non-haematological toxicity, Grade 3 or higher GvHD or Grade 4 or higher haematological toxicity (graded according to the Common Terminology for Criteria for Adverse Events (CTCAE) version 3.0). Any patient that develops a DLT receives no further treatment on trial. Within each arm, the development of DLT in 3 patients terminates treatment within that arm and the lowest tolerated dose by those 3 patients that did not result in DLT becomes the maximum tolerated dose. Secondary endpoints include leukaemia-free and overall survival as well as analyses of immunological responses. Monitoring for disease recurrence will assess leukaemia-free

survival. Routine bone marrow and peripheral blood studies to assess remission status (morphological, flow cytometric, cytogenetic and molecular where there is a marker of residual disease) and donor chimerism are regularly evaluated according to the trial follow-up protocol. Immunological studies will include assessment of lymphoid subset composition and T-cell repertoire analyses prior to and following therapy as well as planned functional evaluation of *in vitro* lysis of AML target cells and leukaemia-specific cytokine production (IFNγ ELISpot assay).

The RFUSIN2-AML1 trial has been open since 2007, although recruitment occurred in earnest after 2010 due to initial tight restrictions on eligibility (e.g. inclusion of patients undergoing matched related Fludarabine-Busulphan-Campath conditioned allografts only) in the earliest versions of the protocol. In my role as co-investigator for this study, and following successful applications for amendments to the original protocol, I had recruited 4 patients to the study, 2 allocated to the DLI only arm and 2 to the vaccine and DLI arm by March 2013. Preliminary results from these patients (safety data, adverse events, clinical response and evaluation) along with lymphoid subset and TCR repertoire analyses will be presented as part of this thesis.

1.8 Aims of this thesis

The aims of this thesis are:

1. To review disease response and toxicity following therapeutic and pre-emptive DLI administration according to an escalating dose schedule in patients who have undergone TCD RIC HSCT for myeloid malignancies at our institution.
2. To determine whether CASAC in combination with WT1 peptide vaccination induces WT1-specific T-cell expansion and cytotoxic responses in a murine model.
3. To examine the feasibility and clinical safety profile of RFUSIN2-transduced primary AML blasts expressing CD80 and IL-2, administered as a whole cell vaccine in combination with DLI, following recurrent AML post-TCD RIC HSCT. Preliminary analyses of lymphocyte subsets and TCR repertoire following vaccine administration and/or DLI will also be described.

Chapter 2 Materials and Methods

2.1 Materials

Peptides

The following were purchased from Peptide Protein Research Ltd (Farnham, UK) at >95% purity in 5mg aliquots:

- RMFPNAPYL$_{126-134}$ (Class I peptide from WT1 protein, WT1-RMF)
- YMFPNAPYL$_{126-134}$ (Heteroclitic modification of the RMFPNAPYL peptide, WT1-YMF)
- SIINFEKL$_{257-264}$ (Class I peptide from Ovalbumin protein, OVA-SIINF)
- ISQAVHAAHAEINEAGR$_{323-339}$ (Class II peptide from Ovalbumin protein, OVA-ISQ)
- KSSAKXVAAWTLKAAA (PADRE 965.10, pan-HLA-DR epitope)[266]
- PGCNKRYFKLSHLQMHSRKHTG$_{396-417}$ (Long (22 amino acid) Class II peptide from WT1 protein, WT1-PGC)

The following Class I peptides were kind gifts from Dr Linda Barber, the Rayne Institute, Coldharbour Lane, London

- YLLPAIVHI$_{148-156}$ (ATP-dependent RNA helicase)
- NLVPMVATVQ$_{495-504}$ (Cytomegalovirus (CMV) pp65 peptide)

The overlapping peptide pool was acquired from Miltenyi Biotec (Cologne, Germany). The non-clinical grade product was used in experiments although a clinical grade product is available:

- PepTivator® WT1 peptide pool, comprising 113 x 15-mer sequences, with 11 amino acids overlap, covering the complete sequence of the human WT1 protein.

Information provided by Miltenyi Biotec on this product was limited. The 60nmol vial of PepTivator® WT1 peptide pool contained 100μg of each peptide according to the datasheet. There are approximately 113 peptides in the pool giving a total weight of 11300μg/11.3mg of peptide in 1 vial of 60nmol PepTivator® WT1 peptide pool. This calculation was used to determine the volume in which to re-suspend the peptides for dosing (Tables 2-4 and 2-5).

Antibodies & Pentamers

Table 2-1 Anti-mouse antibodies used in murine studies

Test Antibody	Clone	Isotype Control	Manufacturer
αCD3 eFluor® 450	17A2	Rat IgG2b K Isotype Control eFluor® 450	eBioscience, San Diego, USA
αCD8a PerCP Cy5.5	53-6.7	Rat IgG2a K Isotype Control PerCP-Cy5.5	eBioscience, San Diego, USA
αCD19 PE-Cy7	1D3	Rat IgG2a K Isotype Control PE-Cy7	eBioscience, San Diego, USA
αCD25 APC	PC61.5	Rat IgG1 K Isotype Control APC	eBioscience, San Diego, USA
αCD4 FITC	GK1.5	Rat IgG2b K Isotype Control FITC	eBioscience, San Diego, USA
αIFNγ PE	XMG1.2	Rat IgG1 K Isotype Control PE	eBioscience, San Diego, USA /BD* biosciences, Franklin Lakes, USA
αFoxP3 PE	FJK-16s	Rat IgG2a K Isotype Control PE	eBioscience, San Diego, USA
αCD28 antibody (stimulating)	37.51	Not applicable	eBioscience, San Diego, USA /BD biosciences, Franklin Lakes, USA
Low endotoxin, azide-free αCD40 antibody (stimulating)	HM40-3	Not applicable	BioLegend, San Diego, USA

*BD, Beckton Dickinson

The following anti-human antibodies were used during AML cell vaccine production and in follow-up immunophenotyping studies on the RFUSIN2 clinical trial. Internal controls (gating on cells known not to express the marker of interest) were used rather than isotype control stains in these particular studies.

Table 2-2 Anti-human antibodies used in the course of vaccine preparation and for immunophenotyping studies during follow-up on the RFUSIN1-AML1 clinical trial

Test Antibody	**Clone**	**Manufacturer**
αCD80 PE	L307.4	BD biosciences, Franklin Lakes, USA
αCD3 V500	UCHT1	BD biosciences, Franklin Lakes, USA
αCD56 PE Cy5.5	CMSSB	eBioscience, San Diego, USA
αCD19 APC	HIB19	BD biosciences, Franklin Lakes, USA
αCD16 eFluor® 450	CB16	eBioscience, San Diego, USA
αCD4 eFluor® 450	RPA-T4	BD biosciences, Franklin Lakes, USA
αCD45RO APC	UCHL1	BD biosciences, Franklin Lakes, USA
αCD27 FITC	M-T271	BD biosciences, Franklin Lakes, USA
αCD69 PE	FN50	eBioscience, San Diego, USA
α HLA-DR PerCP Cy5.5	G46-6	BD biosciences, Franklin Lakes, USA
αCD25 APC	M-A251	BD biosciences, Franklin Lakes, USA
α FoxP3 PerCP Cy5.5	PCH101	eBioscience, San Diego, USA
α NKG2D APC	1D11	BD biosciences, Franklin Lakes, USA
αDNAM-1 FITC	DX11	BD biosciences, Franklin Lakes, USA
αNKp44 PE	P44-8.1	BD biosciences, Franklin Lakes, USA

The following pentamers were purchased from Proimmune Ltd (Oxford, UK) for use in the murine peptide vaccination studies:

- Control pentamer – specific for the Lymphochoriomeningitis virus (LCMV) nucleoprotein 396-404, (R-PE- FQPQNGQFI/D^b)
- OVA-SIINFEKL pentamer (R-PE-SIINFEKL/K^b)
- WT1-RMFPNAPYL pentamer (R-PE-RMFPNAPYL/D^b)

2.1.1 Adjuvant components

Complete Freund's Adjuvant (CFA)	Sigma-Aldrich, St Louis, USA
CpG ODN 1826 (5'-t*c*c*a*t*g*a*c*g*t*t*c*c*t*g*a*c*g*t*t-3') (*Phosphothiorate modified bases)	SIGMA-Genosys, Sigma-Aldrich, St Louis, USA
Polyinosinic–polycytidylic acid sodium salt (Poly I:C)	Sigma-Aldrich, St Louis, USA
Recombinant mouse Interferon Gamma (IFN-γ)	Peprotech, Rocky Hill, USA

2.1.2 Cell lines

293T/17	American Type Culture Collection, (ATCC) Virginia, USA
T2 (TAP deficient) cells	Kind gift, Dr Linda Barber
U937	ATCC, Virginia, USA

2.1.3 Primer sequences

IL-2/CD80 junction FORWARD primer:	
5'TGTGAATATGCTGATGAGACAGCAACC	Sigma-Aldrich, St Louis, USA
IL-2/CD80 junction REVERSE primer:	
5'TGCGAGTTTGTGCCAGCTCTTCA	Sigma-Aldrich, St Louis, USA
CD80 FORWARD primer:	
5'GGCAACGCTGTCCTGTGGTCA	Sigma-Aldrich, St Louis, USA
CD80 REVERSE primer:	
5'CCTCGTCAGATGGCGCAGA	Sigma-Aldrich, St Louis, USA

2.1.4 Media and solutions

BD GolgiStop™ (containing Monensin)	BD Biosciences, Oxford, UK

Cryosure-DMSO (for GMP procedures)	Wak-Chemie Medical GmbH, Steinbach T/s, Germany)
Dimethyl sulfoxide - Hybri-Max™, sterile-filtered (DMSO)	Sigma-Aldrich, St Louis, USA
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich, St Louis, USA
Phosphate buffered saline (PBS)	Sigma-Aldrich, St Louis, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, St Louis, USA
Fetal bovine serum (FBS)	Life Technologies, Carlsbad, USA
Ficoll-Paque Premium	GE Healthcare Bio-Sciences, Piscataway,USA
FoxP3 Fixation and Permeabilisation Concentrate and Diluent kit	eBiosciences, San Diego, USA
Human albumin	Bio Products Laboratory Limited, Herts, UK
Intracellular fixation buffer	eBiosciences, San Diego, USA
Isoflo® Isofluorane	Abbot Animal Health, Maidenhead, UK
Penicillin & streptomycin solution (10,000 units penicillin and 10 mg streptomycin/mL)	Sigma-Aldrich, St Louis, USA
Permeabilization Buffer (10x)	eBiosciences, San Diego, USA
Roswell Park Memorial Institute medium (RPMI-1640)	Sigma-Aldrich, St Louis, USA
Sodium citrate pH 3; 0.05M 12% (v/v) solution	Sigma-Aldrich, St Louis, USA
Sodium hydroxide 1.0N solution	Sigma-Aldrich, St Louis, USA
Squalene, ≥ 98% purity	Sigma-Aldrich, St Louis, USA
Trypan Blue solution (0.4%)	Sigma-Aldrich, St Louis, USA
Tween® 80 - Cell culture tested	Sigma-Aldrich, St Louis, USA
X-VIVO 15 media	Lonza Biologics, Walkersville, USA

2.1.5 All other kits and reagents

Ammonium chloride (NH_4Cl)	Sigma-Aldrich, St Louis, USA
Annexin V APC	BD Biosciences, Franklin Lakes, USA
Anti-Human HLA-A2-PE antibody, clone BB7.2, mouse IgG2bκ	BD Biosciences, Franklin Lakes, USA
Anti-Rat Igκ/Negative Control (FBS) Compensation particles Set	BD Biosciences, Franklin Lakes, USA
Cell Trace™ (Carboxyfluorescein diacetate succinimidyl ester, CFSE)	Invitrogen, Carlsbad, USA

Diethylaminoethyl (DEAE)-dextran viral complexing agent	GE Healthcare, Amersham, UK
Gentamicin sulphate	Beacon Pharmaceuticals Ltd, East Sussex, UK
HIV-1 p24 Enzyme-Linked Immunosorbent Assay (ELISA) kit	PerkinElmer Life Sciences Inc., Boston, USA
Interleukin-2 Duo Set ELISA kit	R&D systems, Minneapolis, USA
Ionomycin calcium salt from Streptomyces conglobatus, ≥98% purity	Sigma-Aldrich, St Louis, USA
Methanol (99.8%)	Sigma-Aldrich, St Louis, USA
Monensin sodium salt, 90-95% purity	Sigma-Aldrich, St Louis, USA
Nuclease-free water	Promega, Wisconsin, USA
Phorbol 12-myristate 13-acetate, (PMA)	Sigma-Aldrich, St Louis, USA
Potassium bicarbonate ($KHCO_3$)	Sigma-Aldrich, St Louis, USA
Power SYBR Green Master Mix	Life Technologies, Carlsbad, USA
QIAamp DNA Mini Kit	QIAGEN, Hilden, Germany
QIAamp® MinElute® Virus Spin Kit	QIAGEN, Hilden, Germany
QuantiTect® Reverse Transcription Kit	QIAGEN, Hilden, Germany
Recombinant human interleukin 3 (rhuIL-3)	CellGenix, Freiburg, Germany
Recombinant human Stem Cell Factor (rhuSCF)	Amgen, Thousand Oaks, USA

2.1.6 Solutions prepared in-house

1. Red Blood Cell (RBC) Lysis Buffer:

   - 8.26g Ammonium Chloride (NH_4CL)
   - 1 g potassium bicarbonate ($KHCO_3$)
   - 0.037 g EDTA

   The above were dissolved in 1 litre of distilled water. The resulting solution was then passed through a 0.2μM filter (Millipore, Massachusetts, USA) and stored at room temperature.

2. Monensin solution:

   - Reconstituted monensin salt in 99.8% methanol to a concentration of 10mM with subsequent dilution for use in assays (final concentration 3μM per well)
   - Alternatively, BD GolgiStop™ was used in some experiments

2.2 Methods

2.2.1 Retrospective analysis of outcomes following Donor Lymphocyte Infusions (Chapter 3)

The methodology for this chapter is described within the publication included in Chapter 3.

2.2.2 WT1 peptide vaccination using combined adjuvants for synergistic activation of cellular immunity (Chapter 4)

2.2.2.1 Mice

C57BL/6 mice were obtained from Harlan (Indianapolis, USA) and housed in a pathogen-free facility at the Biological Sciences Unit (BSU), Denmark Hill campus, according to Home Office Regulations. Female mice, H-2K^b/H-2D^b MHC haplotypes, 6-8 weeks of age, were used in all experiments. Upon arrival at the facility, mice were allowed to acclimatise to the new environment for 1 week before proceeding with an experiment (unless being culled). Experiments were carried out according to protocols stated in Project License 70/6950. Any mice not culled at termination of an experiment were subsequently culled in a timely manner using a Schedule 1 approved humane method.

2.2.2.2 Assessment of peptide binding to MHC molecules using the Immune Epitope Database

As described in Chapter 1, section 1.6.2 and Table 1-3, a number of WT1 epitopes have been used in peptide vaccinations targeting WT1 in mice and in human clinical trials. WT1-RMF has been used extensively in both mouse and human studies due to good binding affinity for both HLA*02:01 and H-2D^b (section 1.6.2). The longer Class II peptide PGCNKRYFKLSHLQMHSRKHTG, used in the multi-epitope vaccine studies described by Scheinberg's group[190,203], had not been evaluated in mice, although the sequence is identical in the mouse and human WT1 proteins. To assess whether sequences within this peptide might bind the mouse class II molecule (I-A^b in C57BL/6), the peptide was analysed using the Immune Epitope Database (IEDB)[181]. This software uses a combination of approaches to assess the binding potential for epitopes deriving from a particular sequence to bind to candidate Class I and II molecules. The analysis demonstrated 8 sequences deriving from the longer peptide with percentile rank between 29-53 using the consensus method, suggesting moderate binding affinities for I-A^b (Table 2-3). Notably, within this long WT1 peptide, 54 potential Class I peptides, ranging in length from 8-11 amino

acids and binding H-2K^b or H-2D^b were identified. Each of these represents candidate epitopes that could be processed and presented via cross-presentation mechanisms to responder CD8+ T-cells (data not shown).

Table 2-3 Peptides from within the long WT1 peptide showing binding to the C57BL/6 Class II molecule I-A^b and as an example, one of the more common HLA Class II alleles, HLA DRB1*04

Peptide sequence	**Class II allele**	**Percentile rank* by consensus method (IEDB)[181]**
CNKRYFKLSHLQMHS	I-A^b	27.91
GCNKRYFKLSHLQMH	I-A^b	29.44
NKRYFKLSHLQMHSR	I-A^b	30.59
KRYFKLSHLQMHSRK	I-A^b	32.34
RYFKLSHLQMHSRKH	I-A^b	40.95
PGCNKRYFKLSHLQM	I-A^b	45.09
YFKLSHLQMHSRKHT	I-A^b	51.13
FKLSHLQMHSRKHTG	I-A^b	53.2
CNKRYFKLSHLQMHS	DRB1*04:01	2.53
NKRYFKLSHLQMHSR	DRB1*04:01	2.53
KRYFKLSHLQMHSRK	DRB1*04:01	2.53
GCNKRYFKLSHLQMH	DRB1*04:01	2.63
RYFKLSHLQMHSRKH	DRB1*04:01	2.66
YFKLSHLQMHSRKHT	DRB1*04:01	5.15
PGCNKRYFKLSHLQM	DRB1*04:01	6.65
FKLSHLQMHSRKHTG	DRB1*04:01	9.11

*Top (low) percentile binders are deemed good binders.

However, whilst such algorithms are extremely useful as a starting point to assess immunogenicity of candidate peptides, all studies must be backed up by functional assessments for a true evaluation of candidate epitopes with immunizing potential.

2.2.2.3 Vaccine preparation

Vaccine components were prepared as listed in Table 2-4 under sterile conditions, in a Class II microbiological safety cabinet (Howorth Airtech Ltd.) Peptides from Peptide Protein Research were supplied in vials containing 5mg of the particular peptide. Following dissolution in the relevant solvent to achieve the desired concentration, components were evenly mixed by vortexing. Peptides were dissolved until a clear solution was obtained; up to 1μl of sodium hydroxide (1.0N solution) was added to achieve this where required. Peptides were then aliquotted and stored in 0.5ml tubes (Eppendorf, Hamburg, Germany) at -20^{o}C (Leibherr Freezer). Repeated freeze-thaw cycles of aliquots were avoided (maximum 3 per vial). Emulsion was prepared, giving a final concentration of 0.4% v/v Tween 80 and 4.4% v/v Squalene in PBS (e.g. 16μl Tween 80, 176 μl Squalene and 3808 μl PBS for final volume of 4ml). Tween 80 was pipetted into a 15ml Falcon tube followed by Squalene and PBS. Following decontamination of the Sonics Vibra Cell sonicator using 70% Ethanol, the mixture was sonicated for 5 seconds at 100% amplitude before repeating, with 30 seconds rest between sonications. Sonication was repeated up to 4 times until a milky white, even emulsion was formed that did not stick to the sides of the Falcon tube. The emulsion was transferred to ice immediately and then stored in a water bath at 37^{o}C (Clifton unstirred water bath) until use (within 4 hours of preparation).

Sufficient vaccine was prepared in one 1ml tube (Eppendorf, Hamburg, Germany) to vaccinate one more than the total number of mice within a group, i.e. for a group of 5 mice, sufficient vaccine was prepared for 6 mice, to allow for any pipetting/syringing errors. Each frozen vaccine component was allowed to thaw to room temperature and then pipetted into each 1ml tube to give the correct amount of each constituent per vaccine (Table 2-5). An equal volume of emulsion was added to each 1ml tube and then mixed thoroughly by vortexing (Labinco L46 vortex) for 60 seconds per tube. Where Complete Freund's adjuvant (CFA) was used instead of CASAC, this was required only for the first vaccination. The glass vial containing CFA was vortexed thoroughly and the required volume of adjuvant was then removed using a pipette and transferred into the 1ml tube already containing the target peptide(s) in PBS. This was then

vortexed thoroughly to ensure an even, milky emulsion was formed. For all subsequent vaccinations in mice primed at first vaccination using CFA, emulsion (0.4% v/v Tween 80, 4.4% v/v squalene in PBS) was used instead of CFA and combined with target peptide(s) in PBS.

Table 2-4 Preparation of peptides and CASAC components

Component	Preparation & storage	Stock concentration
Single peptide sequences: **RMFPNAPYL$_{126-134}$** **YMFPNAPYL$_{126-134}$** **SIINFEKL$_{257-264}$** **ISQAVHAAHAEINEAGR$_{323-339}$** **KSSAKXVAAWTLKAAA(PADRE 965.10)^{266}** **PGCNKRYFKLSHLQMHSRKHTG$_{396-417}$**	Each dissolved in 10% DMSO and PBS. 1.0N Sodium Hydroxide 1μl used to aid solubility where required Aliquotted into 0.5ml tubes (Eppendorf, Hamburg, Germany) and stored at -20^{o}C (Leibherr Freezer)	8mg/ml
WT1 PepTivator®	Dissolved in 500μl PBS Aliquotted into 100μl volumes in 0.5ml tubes and stored at -20^{o}C	22.6mg/ml
Anti-CD40 Antibody	Used neat from stock	1mg/ml
CpG	Dissolved in nuclease-free water Aliquotted into 100μl volumes in 0.5ml tubes and stored at -20^{o}C	5mg/ml
Poly I:C	Dissolved in PBS Aliquotted into 250μl volumes in 0.5ml tubes and stored at -20^{o}C	10mg/ml
Mouse IFNγ	Dissolved in PBS Aliquotted into 25μl volumes in 0.5ml tubes	100μg/ml
Emulsion: **Squalene 4.4%** **Tween 80 0.4%** **PBS**	Components added and mixed by sonication (see text). Used within 4 hours of preparation.	

Table 2-5 Quantity of each CASAC vaccine component per vaccine, with example volumes for 6 mice

Vaccine component	Stock concentration	Amount per vaccine	Volume for 1 mouse	Volume for 6 mice
Class I peptide	8mg/ml	100μg	12.5 μl	75 μl
Class II peptide	8mg/ml	100μg	12.5 μl	75 μl
WT1 PepTivator®	22.6mg/ml*	282.5μg	12.5 μl	75 μl
Anti-CD40 Antibody	1mg/ml	25μg	25 μl	150 μl
CpG	5mg/ml	25μg	5 μl	30 μl
Poly I:C	10mg/ml	50μg	5 μl	30 μl
Mouse IFNγ	100μg/ml	1 ng	1 μl	6 μl
PBS	-	-	to bring final volume of aqueous components to 50μl	to bring final volume of aqueous components to 300μl

* approximate, based on available information from Miltenyi Biotec

2.2.2.4 Vaccination protocol

Vaccinations were performed at 7-10 day intervals up to 4 times. Mice underwent general anaesthesia using isofluorane inhalation. Up to 5 mice were placed in the anaesthetic chamber and anaesthesia induced using an oxygen flow rate of 2l/min, containing up to 3% isofluorane. After 2-5 minutes, anaesthetised mice were individually transferred to a mat for administration of anaesthetic via a mask apparatus. Both flanks of each mouse were shaved (using a Wella contour clipper) to allow clear access and visibility in the region where the vaccine was to be administered. After spraying the shaved areas with antiseptic (Chlorhexidine, Vetasept) and allowing to dry, intra-dermal vaccinations were administered. Vaccine was drawn up into a 0.3 ml insulin syringe with a 30-gauge needle (Beckton Dickinson, Franklin Lakes, USA). Each flank was grasped between the thumb and index finger, at the same time pulling the exposed skin taught. Fifty microlitres of vaccine were injected intra-dermally per flank, with observation of a skin bleb confirming successful intra-dermal administration. Mice were then allowed to recover and observed until mobile and resuming normal behaviour. For CFA, due to the high viscosity of the vaccine, components were aspirated into a 1ml syringe (Beckton Dickinson) and administered using a 25 gauge (Beckton Dickinson) orange needle.

2.2.2.5 Collection of blood and splenocytes

A No. 10 disposable blade (Swann Morton) was used to nick the tail tip to allow bleeding from the tail vein. Blood was massaged from the tail vein into 1ml tubes already aliquotted with 40µl of sodium citrate 0.05M (12%) anticoagulant solution. A maximum of 100µl of blood was collected at each bleed and animals were bled no more than once in 7 days. To harvest splenocytes for the *in vivo* cytotoxicity assays, up to 5 mice were culled at one time by exposure to a rising concentration of carbon dioxide (CO_2) in a CO_2 chamber. Following cessation of breathing, death was confirmed by dislocation of the neck. The left upper abdomen was sterilized with antiseptic spray (Chlorhexidine, Vetasept) and incised to expose the peritoneum. The spleen was dissected out using 2 pairs of sterile forceps. Spleens from splenocyte donors were combined in a 50ml Falcon tube (Beckton Dickinson) containing 5-10ml of PBS or single spleens were transferred to individual Bijoux tubes (Beckton Dickinson) containing 1-2 ml of PBS.

2.2.2.6 Immunophenotyping of PBMCs and splenocytes in murine vaccination studies

Pentamer staining

Staining was performed as recommended by the manufacturers (Proimmune, Oxford, UK). Each vial of pentamer was stored at 4^{o}C and protected from light exposure. Prior to use, the vial was centrifuged at 14,000×*g* (Rotanta 460R centrifuge) for 5-10 minutes and stored on ice until use. Aliquots of 10µl R-PE-RMFPNAPYL/D^{b} pentamer were pipetted into test wells of a labeled 96-well U-bottom plate (Greiner Bio One, Frickenhousen, Germany). R-PE- SIINFEKL/K^{b} pentamer, in 2µl aliquots, was added to 40 µl of whole blood in wells of a 96 well plate. R-PE- SIINFEKL/K^{b} Pentamer was titrated with antigen specific OT-1 splenocytes to saturation point to allow a reduction in the volume used per test (data not shown). 10 µl of R-PE-FQPQNGQFI/D^{b} pentamer (specific for the LCMV nucleoprotein 396-404) were used (per test) to provide an irrelevant control pentamer; mice kept in the pathogen-free facilities should not have been exposed to this virus. In some experiments, irrelevant control pentamers were either R-PE-RMFPNAPYL/D^{b} or R-PE- SIINFEKL/D^{b} in mice immunized with OVA-SIINF or WT1-RMF respectively.

Following pipetting of pentamer into the relevant wells, 40 μl of blood from each mouse was transferred into appropriate wells and left at room temperature, in the dark for 20 minutes. After 20 minutes, test and control antibody stains were set up as per Table 2-6 and left to stain for a further 20 minutes at room temperature. After incubation, 150μl of red blood cell (RBC) lysis solution was added to each well followed by thorough re-suspension. After 10 minutes, the plate was centrifuged at 560-620*g* for 5 minutes. Supernatants were aspirated and a further 200μl of RBC lysis solution was added to each well and re-suspended. Again, the samples were left for 10 minutes before centrifugation at the same settings and aspiration of the supernatant. Following this, cells were washed twice as above with 200μl of PBS before re-suspending in 200μl of PBS to read on the flow cytometer (FACS Canto II, Beckton Dickinson, Franklin Lakes, USA).

Table 2-6 Antibody cocktails used for assessment of T-cell responses to vaccination

Sample type	Antibodies	Volumes
Isotype control for eFluor® 450 PerCP Cy5.5 PE-Cy7	Rat IgG2b κ Isotype Control eFluor® 450 Rat IgG2a κ Isotype Control PerCP-Cy5.5 Rat IgG2a κ Isotype Control PE-Cy7	2μl 1μl 1μl
Pentamer Control cocktail	αCD3 e450 αCD8α PerCP Cy5.5 αCD19 PE-Cy7 R-PE- FQPQNGQFI/D^b) OR R-PE-OVA-SIINFEKL/K^b) OR R-PE-WT1-RMFPNAPYL/ D^b)	2μl 1μl 1μl 10μl 2μl 10μl
Test pentamer	αCD3 e450 αCD8α PerCP Cy5.5 αCD19 PE-Cy7 R-PE-SIINFEKL/K^b) OR R-PE-RMFPNAPYL/ D^b)	2μl 1μl 1μl 2μl 10μl

Intracellular staining for FoxP3 expression: identification of Tregs.

In a new 96-well U-bottom plate anti-CD4 and anti-CD25 antibodies (or isotype control antibodies) were added as described in Table 2-7. Forty microlitres of whole blood from each mouse was transferred into the relevant test and control wells. The plate was left to incubate in the dark for 30 minutes. Fix and Permeabilization buffer was prepared as per the manufacturer's instructions (eBiosciences, San Diego, USA) and stored on ice. As for the surface staining described above, 2 rounds of RBC lysis and washing were performed. After the second wash, the supernatant was removed and replaced with 200μl of fixation and Permeabilization buffer per well and resuspended thoroughly. The plate was then left for a minimum of half an hour at 4^{o}C. After incubation, the plate was centrifuged at 560*g* x 5 minutes and the supernatant aspirated. The wells were then washed twice in 200μl of 1x Permeabilization buffer. After the second wash, the wells were resuspended in 200μl of 1x Permeabilization buffer per well, to which was added 1.5μl of either the anti-FoxP3 PE antibody or the isotype control PE antibody (Table 2-8) followed by thorough resuspension. The plate was left to incubate for 30 minutes at 4^{o}C before washing once in 1x Permeabilization buffer, once in PBS and then being resuspended in 200μl PBS for analysis on the flow cytometer.

Table 2-7 Antibody volumes per well for control and test samples (quantification of Treg frequencies)

Sample type	Antibodies	Volumes
Isotype control for APC FITC	Rat IgG1 K Isotype Control APC Rat IgG2b K Isotype Control FITC	1µl 1µl
Isotype control for FoxP3	Rat IgG1 K Isotype Control PE* αCD4 FITC αCD25 APC	1.5µl 1µl 1µl
Test wells	αCD4 FITC αCD25 APC αFOXP3 PE*	1µl 1µl 1.5µl

*Administered after fixation and permeabilisation

Intracellular staining for IFNγ

Following 5-hour incubation (see *in vivo* cytotoxicity assay, 2.2.2.7), splenocytes were centrifuged at 560*g* for 5 minutes. After aspiration of the supernatant, 200μl PBS was added to each well and washed again (centrifuged at 560*g* x 5 minutes). In a new 96-well U-bottom plate anti-CD3 and anti-CD8 antibodies (or isotype control antibodies) were added as described in Table 2-8. Following centrifugation, supernatants were removed from the culture plate; each well was re-suspended in 100μl of PBS and then transferred to the matching position in the staining plate. The staining plate was incubated for 20 minutes in the dark at room temperature. After 20 minutes, each well was topped-up with 100μl PBS and the plate was spun at 560*g* x 5 minutes. Following centrifugation, supernatants were aspirated and each well resuspended in 200μl of PBS before a further wash (same settings). 100μl of PBS was added to each well and then the contents transferred to individual, appropriately labelled equivalent FACS tubes (Beckton Dickinson, Franklin Lakes, USA). Cells were fixed by slowly adding 100μl of cold intracellular fixation buffer whilst vortexing each tube. The total volume per tube was maintained at approximately 200μl, as per manufacturer's protocol. Tubes were incubated in the dark at room temperature for 20 minutes, after which, without washing, 2ml ice-cold 1xPermeabilization buffer (diluted in deionised water) was added to each tube. Tubes were then centrifuged at 300-400*g* x 5 minutes. After discarding the supernatants, cell pellets were re-suspended in 2 ml of 1X Permeabilization Buffer and centrifuged at 300-400*g* x 5 minutes. Once supernatants had been discarded, each well was re-suspended in 100 μl of 1X Permeabilization Buffer. A fresh staining plate was prepared and 2.5 μl of either the Rat IgG1K Isotype Control PE or αIFNγ-PE antibodies was added to the appropriate wells. The relevant 100μl volume samples were transferred to the respectively labeled wells in the staining plate containing PE antibodies. The plate was then left in the dark for 20 minutes. After incubation, 100μl of 1X Permeabilization Buffer was added to each well and again spun at 300-400*g* x 5minutes. Cells were washed once more at the same settings in 200μl of 1X Permeabilization Buffer followed by 200μl of PBS before re-suspending in 200μl of PBS to analyse on the flow cytometer.

Table 2-8 Antibody volumes per well for control and test samples (intracellular IFNγ assay)

Sample type	Antibodies	Volumes
Isotype control for eFluor® 450	Rat IgG2b κ Isotype Control eFluor® 450	2μl
PerCP Cy5.5	Rat IgG2a κ Isotype Control PerCP-Cy5.5	1μl
Isotype control for PE	Rat IgG1 κ Isotype Control PE*	2.5μl
	αCD3 e450	2μl
	αCD8α PerCP Cy5.5	1μl
Test wells	αCD3 e450	2μl
	αCD8α PerCP Cy5.5	1μl
	αIFNγ PE*	2.5μl

*Only added after fixation and Permeabilization steps have been completed

T2 stabilisation assay

TAP-deficient T2 cells were plated in a 24-well plate (Beckton Dickinson, Franklin Lakes, USA), 1ml per well at a concentration of $5x10^5$ cells/ml in 10% FCS/RPMI. Known HLA-A2 binding peptides (kind gifts from Dr Linda Barber) were $YLLPAIVHI_{148-156}$ (derived from ATP-dependent RNA helicase) and $NLVPMVATVQ_{495-504}$ (CMV pp65 peptide). These peptides had been reconstituted in 10%DMSO/PBS at a stock concentration of 20mg/ml. Similarly, WT1-RMF and OVA-SIINF peptides were each reconstituted at 20mg/ml in 10%DMSO/PBS. Five microlitres of control (known HLA-A2 binders) and test peptides (each at a stock concentration of 20mg/ml) were added to wells containing T2 cells as follows:

T2 cells alone

T2 cells + 10%DMSO/PBS (no peptide)

T2 cells + WT1-RMF (freshly prepared)

T2 cells + WT1-RMF (previously prepared, frozen and thawed)*

T2 cells + OVA-SIINF

T2 cells + YLLPAIVHI

T2 cells + NLVPMVATVQ

*to compare whether frozen and then thawed WT1-RMF had similar ability to stabilise HLA-A2 expression on T2 cells as freshly prepared WT1-RMF.

The plate was incubated overnight at 37^{o}C with 5% CO_2. Contents of each well were transferred to 7 individually labelled FACS tubes and washed twice at 300-400*g* x 5 minutes in PBS before resuspending in 0.1ml PBS. Twenty microlitres of mouse anti-human HLA-A2-PE antibody (BD Biosciences, Franklin Lakes, USA) was added to each well and left to incubate in the dark for 30 minutes at room temperature. After 2 washes in PBS (same settings), the samples were resuspended in 100 μl of PBS and analysed on the BD FACS Canto II machine.

Compensation set-up for Fluorescence-activated cell sorting (FACS)

Compensations were set up at least monthly using Anti-Rat Ig/Negative Control (FBS) Compensation Particles Set (Beckton Dickinson). 100μl aliquots of PBS were added to 7 individually labelled FACS tubes (Beckton Dickinson). After vortexing, one drop of BD CompBeads Negative Control (FBS) followed by 1 drop of the BD^{TM} CompBeads Anti-Rat Ig beads was added to each tube and vortexed. One tube was used as the unstained control; appropriate volumes of rat antibodies fluorescently labeled with PE, PE-Cy7, FITC, e450, PerCP Cy5.5 and APC were added to individual tubes (one fluorochrome-labelled antibody per tube). Tubes were left to stain at room temperature for 20 minutes; after this, compensation samples were washed twice in 1ml PBS per tube. Compensations were then set-up using the in-built programme in FACS Diva.

Flow cytometer

A 3-laser FACS Canto II (Beckton Dickinson, Franklin Lakes, USA) was used for all flow cytometry studies. The high-throughput sampler (HTS) facility was used for plate-based samples. All samples were acquired using the FACS Diva programme (Beckton Dickinson, Franklin Lakes, USA). All samples were analysed using FLOWJO software (Treestar Inc, Oregon, USA).

2.2.2.7 *In vivo* Cytotoxicity Assay

Healthy spleens were harvested from female C57BL/6 mice as described above. One spleen yielded approximately 4-5x10^7 splenocytes, therefore an appropriate number of mice were sacrificed as required for a particular experiment. After harvesting, excess PBS was removed from the 50 ml Falcon tube and the spleens transferred into a Petri dish containing sufficient PBS to cover its base. The spleens were mashed using the base of a ridged plunger from a 10ml syringe (Beckton Dickinson). The spleen pulp mix was passed through a 40μM cell strainer (Beckton Dickinson) into an empty 50ml Falcon tube. Cells were washed in PBS (620*g* x 5minutes) and then resuspended in 10-15ml of sterile filtered RBC lysis buffer. Cells were then washed twice in PBS at the same settings. The cell pellet was re-suspended in 10ml of X-VIVO 15 and a cell count performed using a 1:10 dilution in Trypan Blue (Sigma-Aldrich) in a haemocytometer. Cells were re-suspended to a concentration of 1-2x10^7 splenocytes/ml and then distributed equally into 2x 50 ml Falcon tubes. These 2 tubes were to hold the peptide loaded (target) cells and the non-peptide loaded (non-target) cells respectively. In some experiments, different sets of peptide loaded target cells were required (i.e. OVA-SIINF-loaded splenocytes and WT1-RMF-loaded splenocytes) – cell numbers were adjusted accordingly per tube depending on the number of recipient mice. However, the cell concentration was maintained the same between all tubes.

10μg/ml of the relevant peptide was added to the peptide loaded (target) tube(s). All tubes were left in the incubator at 37^{o}C for 1 hour with gentle shaking to redistribute the peptide every 15 minutes. After 1 hour, X-VIVO 15 was added to each tube, which was then centrifuged at 620*g* x 5minutes. Sterile Fetal Bovine Serum (FBS, Life Technologies, Carlsbad, USA) was added to sterile PBS to create a 5% v/v FBS solution in PBS. Cells were re-suspended in 5% v/v FBS/PBS and washed once. They were then re-suspended in the same volumes used earlier to maintain a concentration between 1-2x10^7 splenocytes/ml. A solution of 5mM CFSE was prepared in Dimethylsulfoxide (DMSO) and added to each peptide-loaded tube such that the final CFSE concentration was 0.3mM and to the non-target cells to reach a final concentration of 3mM CFSE. Cells were incubated for up to 7 minutes and then washed in 5%FBS/PBS twice, followed by a further wash in PBS. Following the final wash, cells were re-suspended at a concentration of 1x10^8 splenocytes/ml. 100μl of cells from each tube were added to 1900μl of PBS in separate Bijou tubes. FACS tubes were prepared containing mixtures of known proportions of CFSE “low”

and “high”-labelled cells to evaluate the CFSE staining and allow any adjustment in final volumes injected to enable approximately 50:50 of “low” and “high” cells in each injection. The target and non-target cells were analysed on the FACS machine to confirm that 2 separate CFSE-labelled populations (“low” and “high”) were discernible. The voltage threshold for FITC was adjusted to ensure that the peak of the CFSE low population was located roughly between 10^3-10^4 and that the peak of the CFSE high population was between 10^4 and 10^5. Prior to injection, peptide-loaded and unloaded cells were mixed. A 1ml syringe (Beckton Dickinson) was used to draw up the splenocytes and 100μl ($1x10^7$ cells) were injected into the tail vein of each mouse using a 30G yellow needle (Beckton Dickinson), taking care to regularly re-suspend the cells.

Eighteen hours later, mice were culled and splenocytes harvested. One ml of PBS was added to wells of a 24 well plate labelled for each group and mouse. One spleen was transferred to the appropriate well and then mashed using a 1 or 2ml syringe (Beckton Dickinson). Each well was filled with PBS and up to 2ml of cells were transferred from each well to a respectively labelled 15ml Falcon tube, avoiding any sediment. Cells were washed in PBS at 620*g* x 5 minutes. After discarding the supernatant, cells were lysed using 500μl of filtered RBC lysis buffer and then washed again in PBS (same settings). Splenocytes were re-suspended in 10ml PBS per tube and 750-1000μl of cells were transferred into respectively labelled FACS tubes and read on the FACS machine to determine the percentages of CFSE “low” and “high” cells. A minimum of 2000 events in the CFSE-labelled population was recorded. The percentage lysis of peptide-loaded cells was determined using the following equation:

% lysis = 1 - [(no. of targets/no. of control cells in immunized animal)/(no. of targets/no. of control cells in unimmunised animal)] X 100.

2.2.2.8 *In vitro* stimulation of splenocytes for the intracellular IFNγ assay

Cell counts were performed on the splenocyte samples prepared as per 2.2.2.7 using Trypan Blue (Sigma) in a haemocytometer to determine viable cells. Cells were then centrifuged at 1900 RPM x 5 minutes and

re-suspended in an appropriate volume of Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich) to give a final concentration of $5x10^6$ cells/ml. A 96-well U-bottom culture plate was prepared, with test wells containing splenocytes from individual mice and a peptide stimulus or control irrelevant peptide, along with 3-5 wells of each of the following types of control:

1. Unstimulated cells

2. Cells to be used for staining with PE isotype control

3. Cells to be used for staining with e450 and PerCP Cy5.5 isotype controls

4. Cells for *in vitro* stimulation with Phorbol 12-myristate 13-acetate (PMA) and Ionomycin (both Sigma)

One million cells (200μl) were plated per well containing 3mM Monensin (Sigma or BD GolgiStop™), 1μg/ml αCD28 antibody (Beckton Dickinson or eBiosciences) and 1μg/ml of the stimulating peptide. Cells used for positive controls were separately re-suspended, in medium containing 20ng/ml PMA and 1μg/ml Ionomycin, along with Monensin at a final concentration of 3mM. After culturing for 5 hours at 37°C, the protocol for intracellular IFNγ staining was followed as outlined in 2.2.2.6.

2.2.2.9 Statistical analyses

All data were analysed using GraphPad Prism software version 5 (GraphPad software, San Diego, California, USA). All analyses required evaluation of 3 or more groups of mice therefore One-way Analysis Of Variance between groups (ANOVA) was employed. Post hoc tests were performed using Tukey's Multiple Comparison test. Any two-tailed P values <0.05 were considered significant.

2.2.3 Whole cell vaccination using CD80/IL-2 modified AML blasts in patients with relapsed AML post allogeneic HSCT (Chapter 5)

2.2.3.1 Patients and treatment assignment

Full details of patient eligibility, treatment and clinical monitoring are provided in the latest version of the clinical trial protocol included in Appendix C. All patients screened for, or recruited to, the clinical trial gave informed consent according to the declaration of Helsinki for trial related-procedures, as per the ethically approved, latest version (3.0) of the clinical trial protocol.

In brief, all patients > 18 years of age with a diagnosis of AML according to WHO criteria (Appendix A) were considered eligible for cryopreservation of AML blasts. Patients who had undergone allogeneic HSCT and experienced morphological relapse of AML with >5% blasts in the bone marrow aspirate and ≥ 25% donor CD3+ T-cell chimerism were also eligible for blast cryopreservation. AML patients undergoing any type of allogeneic HSCT could be recruited; details of relevant conditioning regimens are listed in Appendix B. All but 4 of the screened patients underwent transplantation at King's College Hospital; those patients transplanted at King's College Hospital had undergone *in vivo* lymphodepletion with either alemtuzumab or anti-thymocyte globulin (ATG) according to the regimen. GvHD prophylaxis immediately post-HSCT consisted of cyclosporine 1.5 mg/kg i.v. twice daily (converted to oral route after recovery of oral intake) starting on day -1, titrated according to plasma trough levels (range, 150- 200 ng/L), and tapered from day +56 onward.

Suspected AML relapse post-HSCT was confirmed by bone marrow aspirate and trephine biopsy. Bone marrow aspirate samples underwent morphologic, immunophenotypic, cytogenetic and chimerism analyses. Up to fifty millilitres of bone marrow aspirate (where possible) and 50ml of peripheral blood were collected in EDTA-containing vacutainer tubes (Beckton Dickinson) for isolation and cryopreservation of mononuclear cells (cryopreservation procedures described in 2.2.3.2) from eligible, consenting patients. Blood samples collected contemporaneously with bone marrow and blood harvests were sent to the South London Specialist Virology Centre laboratories at King's College Hospital, London for virology screening (tests described in 2.2.3.4).

Following confirmation of relapsed AML, patients underwent salvage chemotherapy according to the treating physician's discretion. Details of the chemotherapy protocols used are supplied in Appendix B. Patients were reviewed at King's College Hospital upon clinical and haematological recovery (neutrophil count >1.0 $x10^9$/L) and underwent repeat bone marrow evaluation as described above to confirm morphological remission (<5% blasts in the bone marrow aspirate, confirmed by trephine histology). Those patients who had achieved a morphological remission were offered entry into the clinical trial following confirmation that inclusion and exclusion criteria for enrolment were satisfied (summarised below and listed in full in the clinical trial protocol, Appendix C:

Inclusion criteria

1. Patients must be able to give written informed consent and be willing to comply for the duration of the study
2. Sufficient blasts must have been stored to enable a minimum of 3 doses of vaccine to be produced
3. Patients need to have achieved morphological remission (as defined by < 5% blasts in the bone marrow) following cytoreductive chemotherapy for treatment of disease relapse
4. Patients must have recovering lymphocyte count of > $0.5x10^9$/L following cytoreductive chemotherapy
5. Patients must be HIV-negative at relapse
6. Patients must not have active viral infection including Human T-lymphotropic Virus (HTLV-1), hepatitis B or C
7. Patients must have adequate renal and liver function confirmed by: creatinine clearance/ estimated glomerular filtration rate (eGFR) >30mls/min; bilirubin <3.0 x upper limit of normal; Aspartate aminotransferase (AST) <3.0 x upper limit of normal
8. Patients must demonstrate a performance status of 2 or less by Easter Cooperative Oncology Group (ECOG) criteria or >60% by the Karnofsky score (defined in the clinical trial protocol)
9. Women of childbearing potential (WCBP) must have a negative serum or urine pregnancy test within 10 – 14 days of study treatment and on repeat, prior to study treatment

Exclusion criteria

1. Life expectancy of <24 weeks
2. Evidence of graft versus host disease
3. Concurrent use of other forms of anti-leukaemic therapy for relapse
4. Other active malignancy with the exception of carcinoma in situ
5. Significant history of heart disease (unstable angina, myocardial infarction within the past six months)
6. Positive pregnancy test

Patients were allocated in a non-random, alternating manner into either of the trial arms, arm A (escalating doses of DLI) and arm B (escalating doses of DLI and AML Cell Vaccine, ACV), Table 2-9. Doses of DLI with/without ACV were administered at strict 6-week intervals and without delay unless dose-limiting toxicity or other clinically significant event requiring investigation necessitated withholding of treatment until investigated and re-commencement of therapy on trial approved by the Principal Investigator (PI).

Table 2-9 Study treatments according to assignment arm

Dose number	**Arm A treatment: DLI**	**Arm B treatment: DLI & ACV**
Dose 1	$5x10^5$ CD3+ cells/kg	$5x10^5$ CD3+ cells/kg $0.5\text{-}1x10^5$ ACV cells in 250μl carrier medium
Dose 2	$5x10^6$ CD3+ cells/kg	$5x10^6$ CD3+ cells/kg $0.5\text{-}1x10^6$ ACV cells in 500μl carrier medium
Dose 3	$1\text{-}5x10^7$ CD3+ cells/kg	$1\text{-}5x10^7$ CD3+ cells/kg $0.5\text{-}1x10^7$ ACV cells in 1ml carrier medium
Dose 4*	$0.5\text{-}1x10^8$ CD3+ cells/kg	$0.5\text{-}1x10^8$ CD3+ cells/kg $0.5\text{-}1x10^8$ ACV cells in 1ml carrier medium

* where available, (applies to each arm)

Unless stated otherwise, all manufacturing procedures were carried out under Good Manufacturing Practice (GMP) conditions by Dr Lucas Chan PhD, King's College London, in the Rayne Cell Therapy Suite under provisions granted to the facility by the Medicines and Healthcare products Regulatory Agency

(Manufacturer's Authorisation for Investigational Medicinal Products (MAIMP), license 14523) and the Human Tissue Authority (license 11023).

2.2.3.2 Lentiviral vector and vaccine production

Lentiviral vector construct

Development of the lentiviral vector was previously described in Chan et al, 2005[258] and finalised in the Investigational Medicinal Product Dossier (IMPD) v4.0. Manufacture was carried out under GMP conditions and in accordance with the Guideline on Development and Manufacture of Lentiviral Vectors (CHMP/BWP/2458/03). Figure 2-1 details a map of the lentiviral construct.

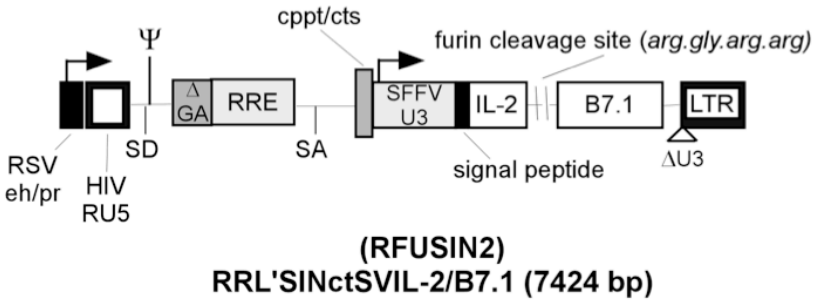


Figure 2-1 The self-inactivating lentiviral vector – RRL'SINctwSVIL-2/B7.1 (RFUSIN2)

Production of the RFUSIN2 lentiviral vector

Large-scale production of the 4 DNA plasmids that form the lentiviral packaging system was carried out by the Bristol Institute for Transfusion Sciences under MAIMP license number 13733. Design and development of the 4 plasmids was previously described[258,267]. A Master Cell Bank (MCB), derived by seeding and propagation of the 293T/17 cell line (American Type Culture Collection (ATCC) Number CRL-11268) in complete medium (10% FBS, 90% Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) 0.05% gentamicin sulphate (Beacon Pharmaceuticals Limited, East Sussex, UK), was generated to serve as the host cell-line to enable large-scale vector production. These cells are referred to as 293T/17 MCB-KCL cells. The 293T/17 MCB-KCL cells underwent sterility testing performed by the commercial company BioReliance (Stirling, UK) and confirmed to be free of viral, fungal and bacterial contamination prior to use

in lentiviral vector production. Furthermore, the company performed DNA fingerprinting to characterise fully the identity of the cells with reference to the DNA profile of HEK 293 cells. BioReliance also excluded the presence of retroviral reverse transcriptase (RT) activity in 293T/17 MCB-KCL cells by fluorescent product enhanced reverse transcriptase assay.

A single batch of the RFUSIN2 virus was prepared for use in all vaccine production for trial subjects. To produce the RFUSIN2 lentiviral vector supernatant, $3x10^7$ 293T/17 MCB-KCL cells were seeded and propagated over 7 days in complete medium, 10% FBS, 90% Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich), followed by transient transfection with the 4-plasmid lentiviral packaging system. After 24 hours of incubation, a primary, lentiviral vector supernatant was harvested. The transfected 293T/17 MCB-KCL cells were replenished with medium to allow a second harvest of lentiviral vector supernatant to be performed a further 24 hours later. The supernatants were concentrated and divided into aliquots before cryopreservation at -80^{o}C, with an aliquot from each batch of concentrate reserved for titration purposes. The RFUSIN2 lentiviral vector also underwent sterility studies performed by BioReliance. Another commercial company GenoSafe (Evry, France) performed characterisation of the lentiviral vector and excluded the presence of HIV-specific recombinants or replication competent lentivirus (RCL). GenoSafe also excluded the presence of residual 293T/17 MCB-KCL cell and plasmid DNA in the lentiviral vector; these analyses were also corroborated by in-house studies to exclude mobilisation and transmission of the RFUSIN2 vector from the AML cell vaccine, detailed in the IMPD.

Titration of RFUSIN2 lentiviral vector

The AML cell line U937 (ATCC Number CRL-1593.2) was used as the indicator cell line for RFUSIN2 titration. U937 cells were inoculated at a cell density of $5x10^5$-$1x10^6$/ml in Roswell Park memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, 1% penicillin and streptomycin (all Sigma, UK) in a 12-well plate. Serial dilutions of the RFUSIN2 vector supernatant were added to the U937 target cells in the presence of 10 μg/ml Diethylaminoethyl (DEAE)-dextran viral complexing agent (GE Healthcare, Amersham, UK). The percentage of cells expressing CD80 in each sample was detected by fluorescence activator cell sorting analysis and the titre of RFUSIN2 determined by the proportion of CD80 positive cells

relative to the number of cells inoculated. Only cells showing CD80 expression between 5-20% were considered representative of single cell transduction by a single vector to allow titre calculation. The total leukaemic cell infectious titre for RFUSIN2 was calculated at $3.5x10^{10}$ i.u. .

Vaccine production

Isolation of peripheral blood and bone marrow mononuclear cells at relapse or diagnosis

Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were isolated by density gradient centrifugation (Ficoll-Paque Premium, GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and subsequently cryopreserved in vapour phase liquid nitrogen in a freezing mixture composed of:

Cryosure-DMSO (Wak-Chemie Medical GmbH, Steinbach T/s, Germany) (10%)

20% w/v human albumin (Bio Products Laboratory Limited, Herts, UK) (18%) and

X-VIVO 15 (72%)

Lentiviral transduction of primary AML blasts

Unselected bone marrow monoculear cells (BMMCs) cryopreserved under GMP conditions for the specific trial subject were thawed from frozen and washed before re-suspension in X-VIVO 15 medium containing 20ng/ml recombinant human stem cell factor (rhuSCF, Amgen, Thousand Oaks, USA) and 10ng/ml interleukin 3 (rhuIL-3, CellGenix, Freiburg, Germany). Cells were cultured under normal conditions ($37^{o}C$ and 5% carbon dioxide (CO_2) concentration) for 18 hours - 3 days. Transduction with RFUSIN2 was performed by inoculation with viral supernatant at an approximate multiplicity of infection (MOI) of 5, in the presence of 5-10mM DEAE-dextran viral complexing agent under normal culture conditions over 18-48 hours, with allowance to extend culture (following replacement with fresh medium) up to 3 further days. Transduced cells were divided for cryopreservation into up to 4 vaccine aliquots (according to the ACV doses described in Table 2-9) in freezing medium as described earlier for initial cryopreservation at blast harvest. Aliquots were stored at $-80^{o}C$ overnight and then transferred to vapour phase liquid nitrogen. During production, cells were kept aside for product validation and sterility analyses.

Product validation and sterility analyses

Upon completion of vaccine production, samples of RFUSIN2-transduced AML cells were sent immediately to Wickham Laboratories (Hampshire, UK) to screen for bacterial contamination and Mycoplasma Experience Limited (Reigate, UK) for *Mycoplasma spp* screening. Product validation was performed to determine CD80 staining and viability by FACS analysis using the FACSCANTO II. Between 0.5-1.5x10^6 RFUSIN2-transduced AML cells were stained with anti-human CD80-PE and Annexin V APC (BD Biosciences, CA, USA). Live cells were gated according to forwards and side scatter parameters to record a minimum of 1x10^4 events. Percentage CD80 expression and mean fluorescence intensity (MFI) of CD80 expression were measured along with the size of the Annexin V APC positive population. Only products demonstrating a minimum of 20% CD80 positive events, with a MFI increased 5-fold above cells stained with a PE isotype control and demonstrating viability of >50% by Annexin-V APC stain could be released for vaccination. For analysis of IL-2 production, a minimum of 5x10^5 RFUSIN2-transduced AML cells were cultured for 24-48 hours, at a concentration of 1x10^6 cell/ml in X-VIVO 15 supplemented with 20 ng/ml rhuSCF and 10ng/ml rhuIL-3. Culture supernatant underwent serial 5-fold dilutions before plating in triplicate in 100μl volumes into a 96-well Enzyme-linked Immunosorbent Assay (ELISA) plate pre-coated with IL-2 capture antibody (IL-2 Duo Set ELISA kit, R&D systems, MN, USA). ELISA was performed as per the manufacturer's instructions, with inclusion of the reference standards provided by the kit. Detection of a minimum IL-2 secretion of 0.1-25ng/10^6cells per 24 hours was required to allow product release (product specifications are detailed in the IMPD).

2.2.3.3 Administration of vaccine and DLI and assessment for dose limiting toxicity

Cryopreserved aliquots of donor lymphocytes were thawed and administered according to institutional protocol by infusion into a peripheral vein. No pre-medication (such as hydrocortisone and chlorpheniramine) were permitted prior to DLI unless agreed by the PI.

The appropriate aliquot of ACV was thawed and irradiated (50 Gy gamma-irradiation) before injection. The entire contents of the vial were drawn into a 1ml syringe and administered using a 25G needle intra-dermally, into as many sites in the lower abdomen as possible, approximately equal to 50-100μl per injection site, 2-3 hours following DLI administration. Patients were monitored for adverse reactions,

development of cytokine release or capillary leak syndromes for a minimum of 24 hours post-injection and then continued follow-up and treatment as per the trial schedule on a 3-weekly basis. Patients were observed for the development of erythema and induration at vaccination sites compatible with a delayed-type hypersensitivity (DTH) reaction[252]. Patients were assessed clinically for development of acute or chronic GvHD following DLI according to recognised criteria[268,269] with histological confirmation by biopsy wherever possible. Adverse events were graded according to the National Cancer Institute Common Toxicity Criteria for Adverse Events, NCI CTCAE version 3.0[270]. Dose-limiting toxicity (DLT) was defined according to the protocol, following the identification of either (1) a Grade 3 (based on NCI CTCAE[270]) or higher non-haematological toxicity that was considered to be vaccine and/or DLI related, (2) prolonged (≥ grade 4 haematological) myelosuppression persisting for more than 4 weeks and confirmed by evidence of a hypocellular marrow (<5% cellularity) with no signs of disease progression (≥5% blasts bone marrow) or (3) Grade 3 or more acute GvHD or moderate chronic GvHD requiring systemic immunosuppression.

Patients developing DLT or exhibiting morphological relapse of disease (≥5% blasts on the bone marrow aspirate, confirmed by trephine histology) were removed from the study and transferred to the follow-up phase of monitoring as per the trial protocol (Appendix C). A cumulative assessment of DLT within each arm was maintained to allow discontinuation of recruitment according to the stopping criteria for DLT detailed in the protocol.

2.2.3.4 Trial monitoring investigations (clinical laboratories)

Blood and/or bone marrow samples were sent to diagnostic laboratories at King's College Hospital according to the clinical trial protocol schedule for evaluation of full blood counts and biochemical status as well as the following specific tests:

Bone marrow morphology, cytogenetics and immunophenotyping, peripheral blood and marrow chimerism

Bone marrow morphology, immunophenotyping and cytogenetic studies on bone marrow aspirates were performed according to standardised protocols as outlined by the European Leukaemia Net guidelines[2]. Standard multi-parametric flow-cytometric evaluation allowed quantification of CD34+ precursors with

phenotypic characteristics of myeloid blasts[2]. Following overnight cultures, a minimum of 20 metaphase cells were analysed for karyotypic evaluation. Fluorescent in-situ hybridization (FISH) analysis was used as an adjunct to chimerism studies in sex-mismatch donor pairs (X- Y- probes) and to detect new or recurrent cytogenetic changes at AML relapse[2]. Chimerism studies were performed on bone marrow and/or blood (unfractionated whole blood and CD3+ and CD15+ fractions) by PCR and fluorescent analysis of short tandem repeat sequences using the Promega Plus PowerPlex 16 System (Promega, Madison, WI).

Bone marrow, liver and skin histology

Bone marrow trephine samples were prepared according to standardised protocols at the diagnostic histopathology lab, King's College Hospital, London. Trephine samples were fixed in zinc formalin decalcified using Shandon TBD-1 Reagents (Thermo Electron, Anatomic Pathology International, Cheshire, United Kingdom). Paraffin sections (4mm) were prepared and stained with haematoxylin and eosin (H & E), Giemsa and Gordon-Sweet silver stain (reticulin analysis). Immunostains were performed to assess for CD34+ and CD117+ populations (both from Dako, Ely, UK). Skin and liver samples were also fixed in zinc formalin with subsequent preparation of paraffin sections as above and stained with H & E. Liver sections were also stained for cytokeratin7 expression (also from Dako, Ely, UK). .

Virology

All peripheral blood samples collected for virological studies were analysed in the South London Specialist Virology Centre Laboratories at King's College Hospital, London. EBV and CMV DNA loads were analysed by real-time quantitative PCR (RQ-PCR) on whole blood according to standardised institutional protocols. Enzyme-Linked Immunoassay (ELISA) was performed on serum for exposure to HIV 1&2, HIV-1 p24 antigen, HTLV 1&2, Hepatitis B and C viruses, *Treponema pallidum*, Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) according to routine diagnostic institutional protocols.

Immunology

Serum samples were sent to the diagnostic Immunology service at King's College Hospital to screen for autoantibodies (anti-nuclear antibodies, anti-double-stranded DNA antibodies, anti-neutrophil cytoplasmic antibodies, anti-smooth muscle antibodies, anti-mitochondrial antibodies, anti-liver kidney

microsomal antibody and anti-gastric parietal cell antibody), presence of a monoclonal immunoglobulin by capillary serum electrophoresis and quantitation of complement components C3 and C4, rheumatoid factor and beta-2 microglobulin according to the trial monitoring schedule.

2.2.3.5 Safety and immune reconstitution assays for monitoring of trial subjects

Drs Yuqian Ma, PhD and Sabine Domning, PhD, King's College London, carried out all trial monitoring safety and immune reconstitution assays according to the protocol schedule. Analysis of peripheral blood immunophenotyping data was performed by myself.

qPCR for detection of vector integration into genomic DNA of patient PBMCs and BMMCs

Genomic DNA (gDNA) was isolated from PBMCs and bone marrow mononuclear cells (BMMCs) at trial monitoring sampling points using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). A set of reference standards were produced in-house by transducing U937 cells with RFUSIN2 lentiviral vector as described in titration of the lentiviral vector, 2.2.3.2. Serial dilutions of the lentiviral vector were again used to allow selection of the population of U937 cells showing approximately 10% expression of CD80 by FACS analysis. gDNA was isolated from both wild type (WT), non-transduced U937 cells and from the selected RFUSIN2-transduced U937 population. Undiluted gDNA from the RFUSIN2-transduced population showing 10% expression of CD80 was considered to have 1 copy of the lentiviral vector per 10 cells. Serial dilutions of gDNA from RFUSIN2-transduced U937 cells in WT U937 gDNA equating to reference standards ranging from 1 lentiviral vector copy/10 cells to 1 lentiviral vector copy/10^6 cells were produced, along with WT U937 gDNA alone as a negative control (absence of the lentiviral vector gDNA). Primers were designed that spanned the junction site encoding the CD80/IL-2 fusion protein and therefore are specific to the lentiviral vector, since they could only be present in RFUSIN2-transduced U937 gDNA; serial dilution of the standards proportionately increased the cycle threshold (Ct). Assay sensitivity was defined as the lowest dilution segregating the Ct (determined at 1 copy in 10^4 cells). Primers recognising CD80 alone recognise gDNA sequences in RFUSIN2-transduced U937, WT U937 and patient cells (positive control). Real-time PCR was performed in a 25μl reaction containing 2μl of gDNA sample or standard, 12.5μl of 2xPower SYBR Green Master Mix (Life Technologies) for detection, distilled water (dH_20) 9.5μl and 1μl of

primers. Primers were diluted 1:10 from stock to form a mixture before adding 1μl to the reaction. qPCR was performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems/Life Technologies, Canada) and settings were fixed: Hold Stage (95^{o}C, 10 minutes), PCR cycles (35 in total) of Melt (95^{o}C, 15 seconds), Anneal (63^{o}C, 15 seconds) and Extend (72^{o}C for 1 minute). A standard curve for detection of the CD80/IL-2 fusion sequence was generated and sensitivity determined based on the lowest dilution of standard whereby sensitivity of qPCR detection of the CD80/IL-2 fusion sequence was lost due to failure of the curves to segregate. All primer sequences are listed in Materials (2.1.3).

qRT-PCR for detection of replicated lentivirus in patient serum

Normal human serum was spiked with a known concentration of RFUSIN2 lentiviral vector; serial dilutions were performed prior to extraction of RNA using the QIAamp® MinElute® Virus Spin Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. cDNA synthesis was performed using the QuantiTect® Reverse Transcription Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. qRT-PCR was performed using the same conditions described for qPCR described above but using primers designed to recognise the sequence encoding wild type (W/T) CD80 in order to generate a standard curve. Assay sensitivity was defined by the lowest dilution of viral vector that segregated Ct; for this set of primers and conditions, this dilution was $3x10^{2}$ copies/ml. Patient serum was treated similarly (isolation of RNA and cDNA synthesis); in addition, 400μl of patient serum was spiked with RFUSIN2 at a concentration of $2.5x10^{4}$ viral copies/ml (i.e. above the sensitivity threshold for the assay) as a positive control. qRT-PCR was performed using the same conditions and primers.

Screening for systemic IL-2 elevation by IL-2 ELISA and for replication competent lentivirus or partial recombinants by p24 ELISA

IL-2 and p24 antigen levels were analysed by ELISA using kits from R&D system, MN, USA and PerkinElmer, MA, USA respectively. ELISA was performed as per the manufacturers' instructions, with inclusion of the reference standards and negative controls provided by the kit as well as healthy donor serum as an additional reference. Supernatant collected from a previously RFUSIN2-transduced U937 cell-line known to produce IL-2 was used as a biological positive control in the IL-2 ELISA. Normal human

serum spiked with RFUSIN2 virus was included in the p24 ELISA as a biological positive control. Quality control indicators included ensuring that R^2 was > 0.98 for the standard curve, that the optical density at 450nm (IL-2 ELISA) and 490nm (p24 ELISA) was >1.0 for wells at the highest concentration of the standards and that consistency was observed between replicates. Assay sensitivity was 20pg/ml for IL-2 and 15pg/ml for p24.

Assessment of immune reconstitution in trial subjects

Immunophenotyping of PBMCs

PBMCs were isolated at the specified trial time points under non-GMP conditions but using the same method described in 2.2.3.4. Cryopreserved PBMCs were thawed rapidly and washed in X-VIVO 15 at 300g for 5 minutes. 2.5-5x10^5 cells were stained with antibody cocktails as described in Table 2-10 and lymphocyte subsets characterised according to expression markers listed in Table 2-11. Examples of flow cytometric analyses are given in Figures 2-2 to 2-6. Compensation controls were prepared using anti-mouse immunoglobulin compensation beads (BD Biosciences) as per the manufacturer's instructions. Intracellular forkhead box P3 (FoxP3) staining was performed using the fixation and permeabilization protocol supplied by the manufacturer (eBiosciences, CA, USA). All samples were analysed on the FACS CANTO II (BD) using FACS Diva software (BD). Results were analysed using FLOWJO software (Tree Star Inc., Oregon, USA).

The lymphocyte subset data derived from eleven healthy, age-matched volunteers were kindly provided by Dr Linda Barber. The median age of the 11 volunteers was 50 years (interquartile range 44-54 years). Drs Katie Matthews and Linda Barber had previously performed the immunophenotyping studies and analyses and the results were presented as part of the study described in Matthews et al[271]. The antibodies used for those immunophenotyping studies comprised: CD4 (clone SK3), CD8 (SK1), CD25 (2A3), CD27 (M-T271), CD45RO (UCHL1), CD56 (B159), (BD Biosciences) and CD3 (OKT3), CD19 (HIB19), FoxP3 (PCH101), and rat IgG2a isotype control (eBR2a) (eBioscience). The definitions of the individual lymphocyte subsets were as described in Table 2-11; however, Tregs were defined as CD4+ CD25high, Foxp3+ (without the use of CD27).

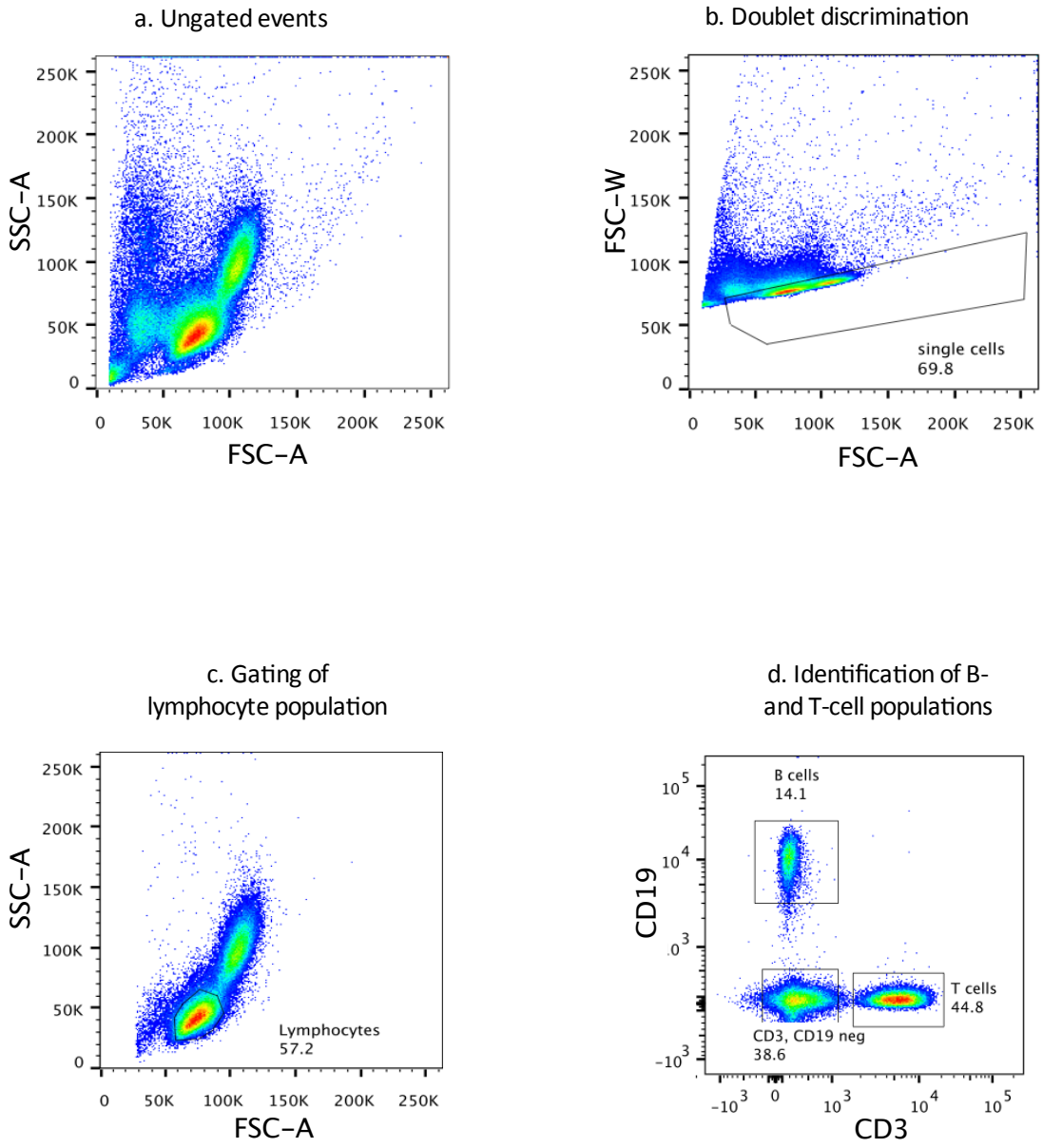
Table 2-10 Antibody cocktails used for immunophenotyping of B-, T- and NK-cell subsets during RFUSIN2-AML1 trial follow-up

Cell subset of interest	Cell marker	Fluorochrome	Clone	Volume of antibody per test (μl)	Supplier
NK- and B-cells	CD3	V500	UCHT1	1	BD
	CD56	PerCP Cy5.5	CMSSB	2	eBiosciences
	CD16	eFluor 450	CB16	2	eBiosciences
	CD19	APC	HIB19	10	BD
T-cell subsets & activation markers	CD3	V500	UCHT1	1	BD
	CD4	eFluor 450	RPA-T4	1	BD
	CD45RO	APC	UCHL1	10	BD
	CD27	FITC	M-T271	10	BD
	CD69	PE	FN50	5	eBiosciences
	HLA-DR	PerCP Cy5.5	G46-6	5	BD
Regulatory CD4+ T-cells	CD3	V500	UCHT1	1	BD
	CD4	eFluor 450	RPA-T4	1	BD
	CD27	FITC	M-T271	10	BD
	CD25	APC	M-A251	5	BD
	FoxP3	PerCP Cy5.5	PCH101	8	eBiosciences
NK-cell activation markers	CD3	V500	UCHT1	1	BD
	CD56	PerCP Cy5.5	CMSSB	2	eBiosciences
	CD16	eFluor 450	CB16	2	eBiosciences
	NKG2D	APC	1D11	20	BD
	DNAM-1	FITC	DX11	20	BD
	NKp44	PE	p44-8.1	20	BD

Table 2-11 Immunophenotypic markers used to identify lymphocyte subsets in RFUSIN2-AML1 trial subjects during follow-up

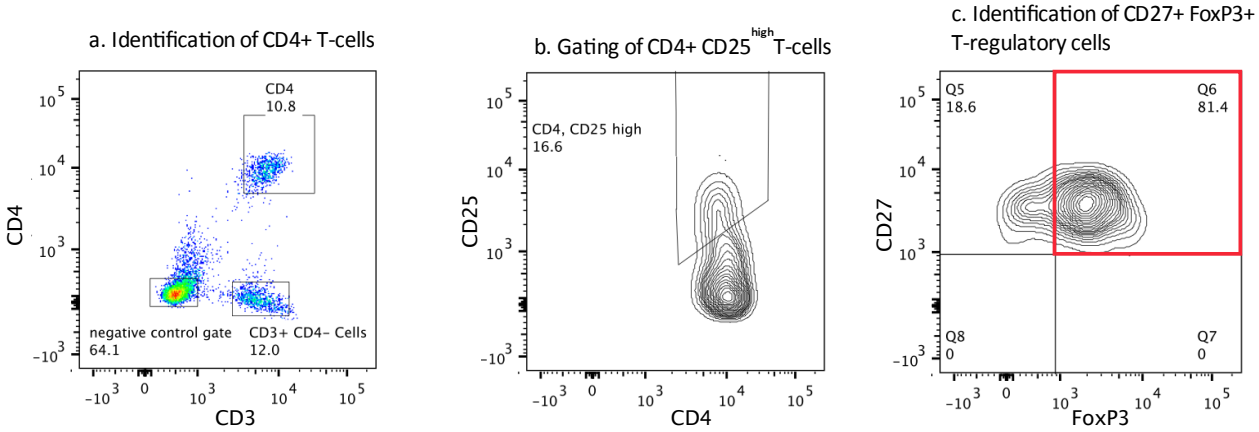
Cell Subset	Phenotype
CD4 T-cell	**CD3+ CD4+**
Activated CD4 T-cell	CD3+ CD4+ CD69+ or HLA-DR+
CD4 naïve	CD3+ CD4+ CD45RO- CD27+
CD4 memory	CD3+ CD4+ CD45RO+ CD27+
CD4 effector	CD3+ CD4+ CD45RO+ CD27-
CD4 terminal effector	CD3+ CD4+ CD45RO- CD27-
CD4 regulatory T-cell	CD3+ CD4+ CD25high CD27+ Foxp3+
CD8 T-cell	**CD3+ CD4-**
Activated CD8 T-cell	CD3+ CD4- CD69+ or HLA-DR+
CD8 naïve	CD3+ CD4- CD45RO- CD27+
CD8 memory	CD3+ CD4- CD45RO+ CD27+
CD8 effector	CD3+ CD4- CD45RO+ CD27-
CD8 terminal effector	CD3+ CD4- CD45RO- CD27-
B-cell	**CD19+ CD3-**
NK-cell	**CD3- CD56+ CD16+/-** CD56 bright CD16 +/- CD56 dim CD16 +/-
NK activation markers	CD3- CD56+ NKG2D+
	CD3- CD56+ DNAM-1+
	CD3- CD56+ NKp44+
	CD3- CD56 bright/dim NKG2D+
	CD3- CD56 bright/dim DNAM-1+
	CD3- CD56 bright/dim NKp44+

Figure 2-2 Representative plots demonstrating the gating strategies used to identify B-cells and T-cell subsets in the peripheral blood of trial subjects and healthy volunteers



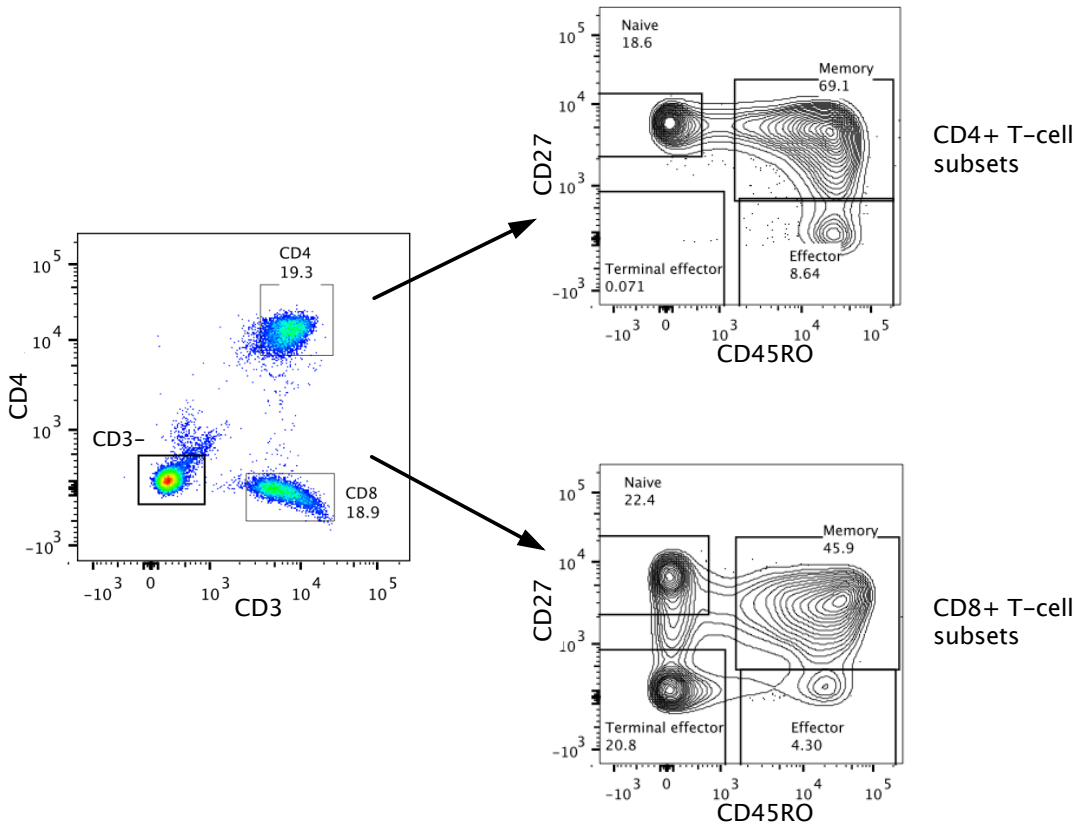
Representative plots demonstrating the identification of peripheral blood lymphocytes and subsets in trial subjects or healthy volunteers. (a-c) Following doublet discrimination, low forward and side scatter properties are used to identify the lymphocyte population. (d) Staining with anti-CD19 and anti-CD3 fluorescent antibodies allows identification of B- and T-cells. Figures given are percentages.

Figure 2-3 Identification of CD4+, CD25^{high}, FoxP3+, CD27+ T-regulatory cells



Following surface staining with anti-CD4, anti-CD25 and anti-CD27 fluorescent antibodies, fixation and permeabilisation of cells according to the manufacturer's protocol was performed. Intracellular staining for FoxP3 expression with an anti-FoxP3 fluorochrome was performed. After gating on lymphocytes (as in Figure 2-2a-c), representative plots show identification of CD4+ T-cells (a), gating of CD4+ CD25^{high} T-cells (b) and CD27+ FoxP3+ T-regulatory cells. Expression of CD27 was used to distinguish T-regulatory cells expressing CD25 and FoxP3 rather than FoxP3-expressing CD25+ effector T-cells[272,273]. Figures given are percentages.

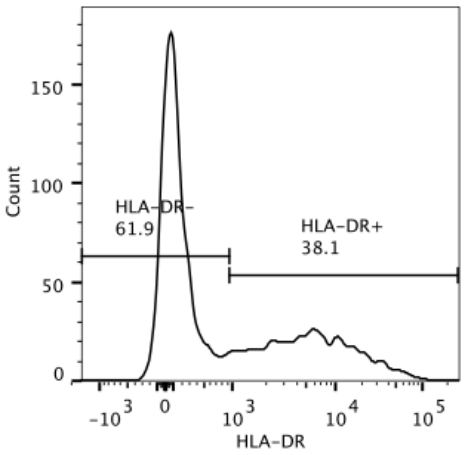
Figure 2-4 Identification CD4+ and CD8+ T-cell subsets



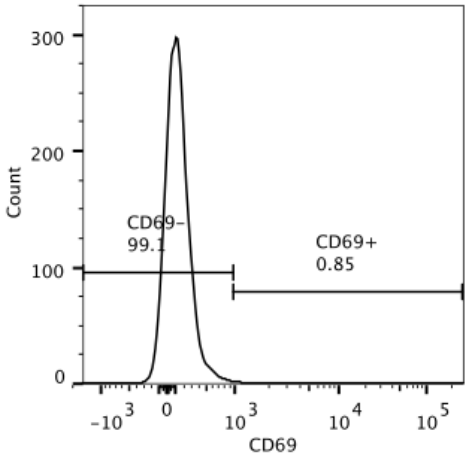
After gating on lymphocytes (Figure 2-2 a-c), surface staining with CD3 was used to identify T-cells, which were then divided by CD4 expression into CD4+ and CD8+ T-cells. CD27 and CD45RO expression was used to identify naïve, memory, effector and terminal effector CD4+ and CD8+ T-cells respectively (see Table 2-11 for description of marker expression by T-cell subsets). Figures given are percentages.

Figure 2-5 Expression of activation markers by CD4+ and CD8+ T-cells

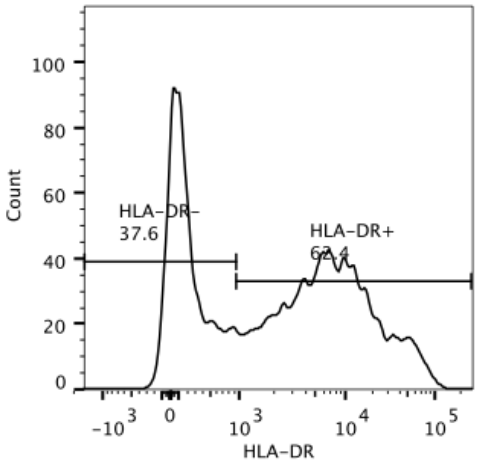
a. HLA-DR expression by CD4+ T-cells



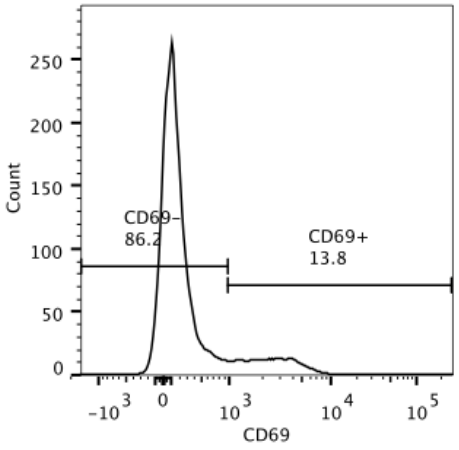
b. CD69 expression by CD4+ T-cells



c. HLA-DR expression by CD8+ T-cells

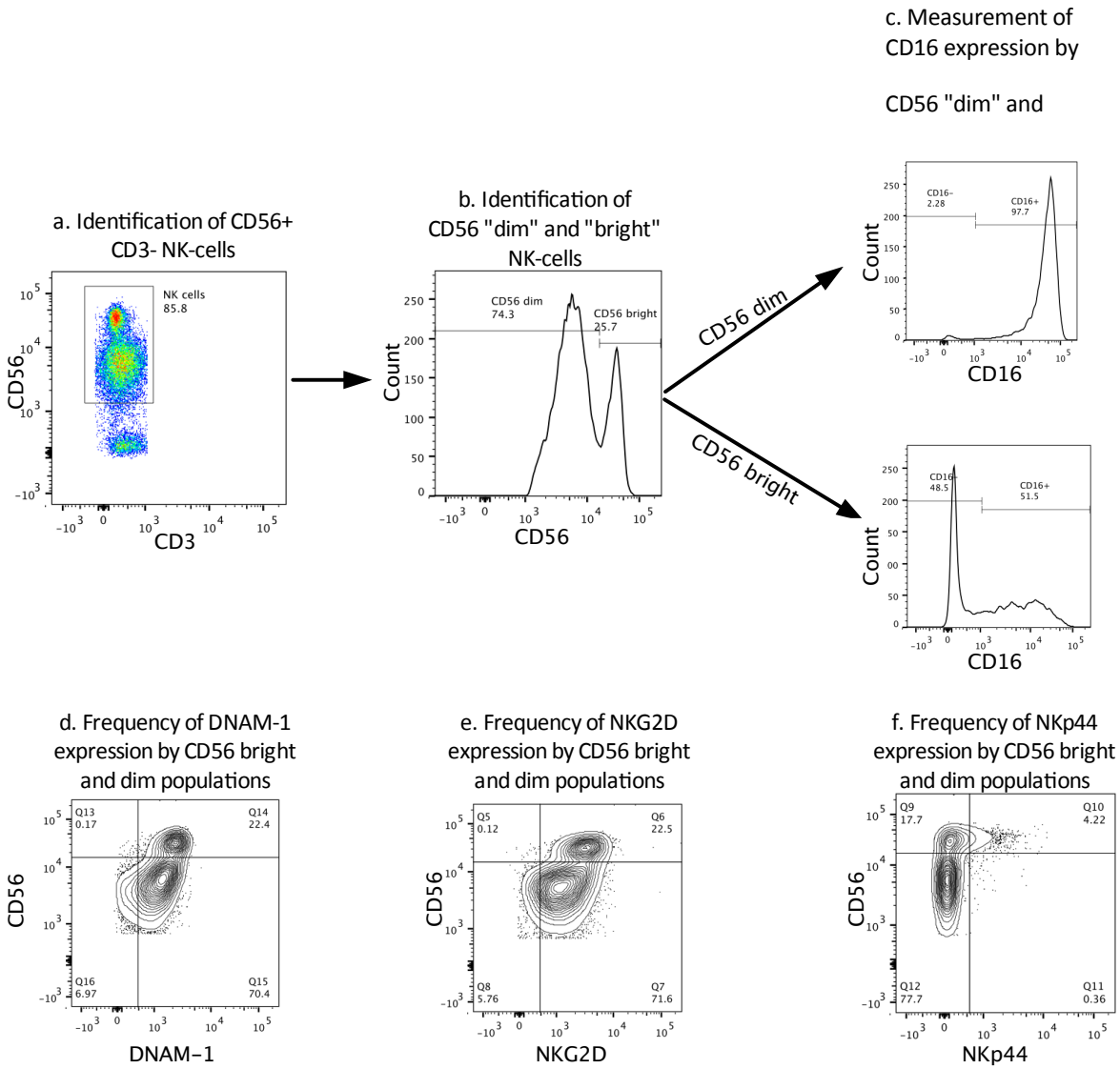


d. CD69 expression by CD8+ T-cells



After identification of CD4+ and CD8+ T-cells (as shown in Figure 2-4), expression of the activation markers HLA-DR (a,c) and CD69 (b,d) was determined for theses two T-cell subsets. Figures given are percentages.

Figure 2-6 Immunophenotypic identification of NK-cell subsets



Following identification of lymphocytes and CD3- CD19- events (Figure 2-2), NK-cells were identified by absent CD3 expression and expression of CD56 (Figure 2-6a). High and low intensity expression of CD56 by these NK-cells allowed distinction into the NK "bright" and "dim" sub-populations (Figure 2-6b) which were shown to have differential expression of CD16 (Figure 2-6c), as previously described[274]. Figures given are percentages.

High throughput sequencing of T-Cell receptor sequences

gDNA samples isolated from PBMCs were sent to Adaptive Biotechnologies (Seattle, USA) to undergo high-throughput sequencing using the ImmunoSEQ assay. This technology was used to permit sequencing of TCR β-chain CDR3 regions using a multiplex PCR assay with established methodology[275,276]. The assay uses 52 forward primers designed to recognise the range of Vβ genes and 13 reverse primers recognising each Jβ gene in order to generate 60 base-pair fragments encompassing the entire range of VDJ combinations. These were then sequenced using the Illumina HiSeq platform and the sequences analysed using ImmunoSeq software tools by Dr David Hamm, PhD at Adaptive Biotechnologies. The total number of reads for each sample was measured and sub-divided into productive, out of frame and stop-codon containing sequences. The number of unique sequences (productive, out of frame and stop codon-containing) was determined following error corrections as described in Robins et al, 2009[275]. A measure of Shannon's entropy for each sample was performed by Adaptive Biotechnologies as a recognised means to analyse clonality within the dataset[277]. Adaptive Biotechnologies used normalized entropy (entropy divided by the log base 2 of the number of productive unique sequences) to determine clonality. The international IMmunoGeneTics (IMGT) database was searched via the ImmunoSeq analysis tools to identify the Vβ and Jβ genes used to produce individual CDR3β sequences[278].

Chapter 3 Outcomes Following Donor Lymphocyte Infusions Post-T-cell Depleted Allogeneic Haematopoietic Stem Cell Transplants for Acute Myeloid Leukaemia and Myelodysplastic Syndromes

3.1 Introduction

Whilst donor lymphocyte infusions (DLI) have been used with considerable success to treat recurrence of chronic myeloid leukaemia post-allogeneic transplantation, their efficacy in the context of other more rapidly progressive myeloid malignancies, such as AML or MDS, is uncertain. The majority of available data regarding outcomes following therapeutic DLI (tDLI) are derived predominantly from retrospective studies of small heterogeneous patient cohorts or combined from transplant registry records. A number of these reports are summarised in Table 1-2. Thus far, outcomes following tDLI for recurrent AML and MDS mainly following myeloablative and/or T-replete HSCT have been unsatisfactory, with survival frequently estimated at less than 25% by 2 years following tDLI and rates of GvHD reaching more than 50%[11,21,130-136]. There have been few reports specifically of outcomes following tDLI for AML and MDS in the context of reduced intensity conditioning (RIC) regimens incorporating T-cell depletion (TCD)[13,139,140]. In one study, while more than half of patients showed complete or partial disease regression following tDLI, rates of GvHD were higher than 50%[139]. Our own preliminary data in AML and MDS patients with recurrent disease following alemtuzumab-containing RIC HSCT suggested response rates to tDLI of greater than 50% confined to those patients with low disease burden, but long-term follow-up data has been lacking[140]. These data are particularly relevant in the context of this thesis, since the use of an AML cell vaccine (ACV) to combine with administration of tDLI to treat recurrent AML post-HSCT aims to boost the induction of GvL activity. However, there remains a risk of triggering harmful GvHD. A clearer understanding of the efficacy and toxicity of tDLI in the absence of co-administration of the ACV is informative with respect to providing benchmarks against which the combination of ACV with tDLI can be evaluated in the future.

Our own data and that from other groups have raised the possibility that DLI may be particularly efficacious where disease burden is low e.g. in the setting of cytogenetic but not morphological disease recurrence[13,140]. This may be further extended to the setting of impending relapse, although identification

of at risk patients is an area of debate. Mixed donor chimerism, particularly in the T-cell depleted HSCT setting, where it is more commonly observed, has been variably linked to increased risk of disease recurrence[139,147,150]. Declining donor T-cell percentages and persistent, predominant recipient T-cell chimerism have been considered indicators to initiate pre-emptive DLI (pDLI) to drive conversion to full donor chimerism and prevent relapse[143,151]. However, there is a lack of consensus regarding the donor CD3 percentage threshold that should trigger therapy, as well as the dosing and scheduling of pDLI. Timing of initiation of pDLI has been a specific area of debate, following a report of greater than 50% GvHD incidence in patients who received DLI at less than 6 months following TCD RIC HSCT[137]. However, in the context of diseases such as AML that may show a rapid tempo of recurrence (often within the first year post-HSCT), early administration of pDLI for low or falling chimerism may be warranted[155]. We have treated a uniform cohort of patients at our institution with pDLI according to an escalating dose protocol, which is sometimes administered before 6 months post-TCD RIC HSCT for AML and MDS. I present the efficacy and toxicity of our approach in this thesis.

I analysed the outcomes following pre-emptive or therapeutic DLI in 113 patients following T-cell depleted RIC HSCT for AML or MDS treated at King's College Hospital between 1999 and 2010. The results of this retrospective study were published in Biology of Blood and Marrow Transplantation, 2013, 19(4): 562-568, and, in accordance with the regulations governing PhD Theses at King's College, London, I have chosen to present this work as published.

3.2 Results

I collected the clinical data for this study using electronic and paper records. I analysed the data that is presented in the publication below using SPSS software. Dr Victoria T. Potter also contributed to data collection and analysis. My statistical analyses were verified independently by Dr Rachel Pearce, who performed the competing risk analyses. I wrote the manuscript, which was critically reviewed and edited by the co-authors and Professor Ghulam J. Mufti. Professor Ghulam J. Mufti and I designed the study outline.

Biol Blood Marrow Transplant 19 (2013) 562–568

Outcome of Donor Lymphocyte Infusion after T Cell–depleted Allogeneic Hematopoietic Stem Cell Transplantation for Acute Myelogenous Leukemia and Myelodysplastic Syndromes

Pramila Krishnamurthy [1], Victoria T. Potter [1], Linda D. Barber [1], Austin G. Kulasekararaj [1], Zi Yi Lim [1], Rachel M. Pearce [2], Hugues de Lavallade [1], Michelle Kenyon [1], Robin M. Ireland [1], Judith C.W. Marsh [1], Stephen Devereux [1], Antonio Pagliuca [1], Ghulam J. Mufti [1,*]

[1] *Department of Haematological Medicine, King's College Hospital, London, UK*
[2] *British Society of Bone Marrow Transplantation, London, UK*

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A B S T R A C T

Relapse occurs in 30%-50% of recipients of T cell–depleted (TCD) reduced-intensity conditioned (RIC) hematopoietic stem cell transplantation (HSCT) for acute myelogenous leukemia (AML) and myelodysplastic syndromes (MDS). Despite limited published supportive data, donor lymphocyte infusion (DLI) is used preemptively (pDLI) to improve donor chimerism and prevent relapse, and therapeutically (tDLI) after disease recurrence. We evaluated the efficacy and toxicity of pDLI and tDLI in 113 patients after TCD (alemtuzumab, n = 99; antithymocyte globulin, n = 14) RIC HSCT for AML or MDS. Recipients of pDLI (n = 62) had an estimated 5-year overall survival (OS) of 80% and an event-free survival of 65%. More than one-half (52%; n = 32) of the patients received pDLI within 6 months post-HSCT; despite this, the 5-year incidence of graft-versus-host disease was only 31% (95% confidence interval [CI], 19%-43%). Recipients of tDLI (n = 51) had an estimated 5-year OS of 40% and a 5-year relapse/progression rate of 69% (95% CI, 54%-81%). Recipients of tDLI at >6 months post-HSCT had a significantly superior 5-year OS after tDLI compared with those treated earlier (P = .008). The cumulative incidence of graft-versus-host disease at 5 years after tDLI was 45% (95% CI, 23%-65%). We demonstrate that pDLI safely promotes durable remission after TCD RIC HSCT for AML or MDS, and that tDLI salvages patients after late relapse with greater efficacy.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) remains the sole curative therapy for patients with intermediate-risk or poor-risk acute myelogenous leukemia (AML) and myelodysplastic syndromes (MDS) [1,2]. Reduced-intensity conditioning (RIC) regimens allow the use of HSCT in older patients and in those with comorbidities by carrying lower nonrelapse mortality (NRM) [3-5]. NRM and morbidity are further improved by T cell depletion (TCD), using alemtuzumab or antithymocyte globulin (ATG), which reduces severe graft-versus-host disease (GVHD) [6,7].

Relapse is the major cause of treatment failure after TCD RIC HSCT [8,9]. Delayed immune reconstitution after RIC with an alemtuzumab-containing regimen might contribute to the relapse risk through a reduced graft-versus-leukemia (GVL) effect [10-12]. To counter this, donor lymphocyte infusion (DLI) is commonly administered after TCD RIC HSCT, which enhances the GVL effect [13,14]. Low or declining donor T cell chimerism may herald impending relapse [15] and guide preemptive DLI (pDLI) to improve chimerism and maintain remission. There is currently no consensus regarding the timing and dosing of pDLI after TCD RIC HSCT for AML/MDS [16,17]. Indeed, an earlier study suggested that pDLI given before 6 months after TCD RIC HSCT induced high rates of GVHD [13]. This is relevant particularly for patients with AML/MDS, who are at greatest risk for relapse at 6-12 months after TCD RIC HSCT [7]. Whether administration of pDLI before 6 months after TCD RIC HSCT is safe and efficacious remains undetermined. After disease recurrence post-HSCT, tDLI may be used alone or in combination with chemotherapy as salvage therapy. Although some studies support tDLI to treat relapse of AML/MDS after T cell–replete or myeloablative HSCT [18-20], data after TCD RIC HSCT is limited [16,17]. Thus, we sought to review outcomes after preemptive and therapeutic DLI administration in patients treated with TCD RIC HSCT for AML and MDS at our institution between 1999 and 2010.

METHODS

Patients

A total of 113 patients treated with DLI after TCD RIC HSCT for AML, MDS, and MDS/myeloproliferative neoplasm (MPN), defined according to the World Health Organization classification scheme [21], were identified. Cytogenetic risk was determined when applicable [22,23]. Pre-transplantation patient characteristics are presented in Table 1. All patients received a fludarabine and busulfan-based RIC regimen (details of regimens are provided in Table S1). The total fludarabine dose was 150 mg/m^2, and total busulfan dose was 6.4 mg/kg i.v. (n = 67), 12.8 mg/kg i.v. (n = 13), 8 mg/kg orally (n = 27), or 16 mg/kg orally (n = 6). In vivo T cell depletion in sibling and unrelated donor transplantations was done using 20 mg alemtuzumab (Campath; Genzyme, Boston, Massachusetts) daily from day -8 to day -4. From 2007, 6 mg/kg (total dose) antithymocyte globulin (ATG; Genzyme) on day -4 to day -1 was used in sibling donor HSCT. For GVHD prophylaxis, cyclosporine (1.5 mg/kg i.v. twice daily, orally after recovery of oral intake) was started on day -1, titrated according to plasma trough levels (range, 150-200 ng/L), and tapered from day +56 onward.

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* Correspondence and reprint requests: Ghulam J. Mufti, Department of Haematological Medicine, King's College Hospital, London, UK.
E-mail address: ghulam.mufti@kcl.ac.uk (G.J. Mufti).

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Table 1
Characteristics of the pDLI and tDLI Cohorts

Characteristic	pDLI (n = 62)	tDLI (n = 51)	*P* Value
Age, years, median (range)	54 (19-69)	55 (29-72)	.09
Sex, male/female, n	29/33	32/19	.09
Conditioning, n			
Fludarabine/busulfan/Campath	56	43	.26
Fludarabine/busulfan/ATG	6	6	
FLAMSA	0	2	
Donor, n			
Sibling	28	23	.57
Matched unrelated	34	28	
Stem cell source, n			
Bone marrow	14	9	.27
Peripheral blood stem cells	48	42	
HLA matching, n			
10/10 match	49	43	.57
8-9/10 match	13	8	
Diagnosis, n			
AML (de novo, secondary)	34 (17, 17)	24 (11, 13)	.28
MDS RAEB I/II	13	15	
MDS RCMD	12	6	
MDS/MPN	3	6	
Cytogenetic risk group, n*			
Good	15 (1/14)	9 (1/8)	.75
Intermediate	29 (22/7)	24 (16/8)	
Poor	8 (4/4)	8 (3/5)	
Not available	10	11	
Disease status at transplantation, n (%)			
<5% blasts	52 (84)	41 (80)	.31
>5% blasts	2 (3)	5 (10)	
Cytogenetic disease	4 (6)	2 (4)	
Missing data	4 (6)	3 (6)	
GVHD post-HSCT, n			
None	54	37	.044
Acute GVHD grade I-II	7	6	
Acute GVHD grade III-IV	0	2	
Chronic GVHD	1	6	
Time to DLI, days, median (range)	176 (87-1304)	277 (74-2801)	<.01
Donor CD3% pre-DLI, median (range)	23 (0-77)	87 (0-100)	<.01
Number of DLI doses, median (range)	2 (1-7)	2 (1-8)	.21
Total DLI dose, $CD3^+$ cells/kg, median (range)	1.5×10^6 (1×10^5-1.66×10^8)	9.5×10^6 (5×10^5-1.43×10^8)	.03

FLAMSA indicates fludarabine/amsacrine/cytarabine; RAEB, refractory anemia with excess of blasts; RCMD, refractory cytopenia with multilineage dysplasia.
* Total number followed by breakdown of numbers in AML/MDS groups. Cytogenetic groups were defined using the International Prognostic Scoring System [22] for MDS and revised Medical Research Council classification for AML [23].

Chimerism Studies and Disease Status Assessments

Chimerism was assessed routinely at days +28, +56, +100, +180, and +365 post-HSCT and at 3- to 6-month intervals thereafter. PCR and fluorescent analysis of short tandem repeat sequences on bone marrow or whole blood and peripheral blood $CD3^+$ and $CD15^+$ cell fractions using the Promega PowerPlex 16 System (Promega, Madison, WI), along with X,Y-fluorescence in situ hybridization in donor–recipient sex mismatches, were used for chimerism assessment. Full donor chimerism (FDC) was defined as donor CD3 percentage (CD3%) ≥95%; mixed donor chimerism (MDC), as donor CD3% of 5%-94%. Relapse was defined as detection of >5% myeloblasts by morphology and/or detection of new/recurrent cytogenetic changes using metaphase and X,Y-fluorescence in situ hybridization analyses.

DLI Administration and Monitoring; Assessment of GVHD

Institutional protocol required that all patients with progressively declining donor CD3 chimerism or relapsed disease post-TCD RIC HSCT receive pDLI or tDLI, respectively. DLI was administered using an escalating dose schedule (Table S2) to patients off immunosuppression and without GVHD. The same dosing schedule was used irrespective of donor type (sibling versus matched unrelated) or degree of HLA-mismatch.

pDLI was administered after day +100 to patients with persistent donor CD3% <50% or declining CD3% (a drop of at least 20% on successive assessments 4-6 weeks apart). Mixed chimeric patients who were on cyclosporine therapy had pDLI withdrawn before confirming the requirement for pDLI by repeat chimerism assessment. The chimerism response to pDLI was determined by the achievement of either sustained FDC or stable MDC (CD3% <95%).

tDLI was administered to patients either alone or after chemotherapy for relapse. In addition to chimerism analysis, bone marrow assessments (morphology, cytogenetics, and immunophenotyping) were performed to evaluate response to tDLI. GVHD (acute [aGVHD] and chronic [cGVHD]) was assessed according to published criteria [24,25], with histological confirmation whenever possible.

Statistical Analyses

Patients were censored at July 1, 2011, or at the date of last known follow-up, death, or second HSCT. Patient, disease, and transplantation-associated variables were compared using the $\chi 2$ test or Fisher exact test for categorical variables and the Mann-Whitney *U* test for continuous variables. Overall survival (OS) after DLI was defined as the interval from first DLI administration to the date of censoring. Event-free survival (EFS) post-DLI was defined as the interval from first DLI until the date of censoring or relapse. Survival curves were estimated according to the Kaplan-Meier method. Two-tailed *P* values <.05 were considered significant. Analysis of cGVHD post-pDLI with respect to chimerism was performed using univariate logistic regression, with chimerism categorized as FDC, MDC, or no sustained improvement in chimerism and treated as ordered categories. Incidence of GVHD was analyzed by competing-risk analysis using the method of Fine and Gray [26], with relapse considered a competing risk with cGVHD. All statistical analyses were performed using Stata version 11.2 (StataCorp, College Station, TX).

RESULTS

A total of 113 patients with AML (n = 58; 50%), MDS (n = 46; 40%), or MDS/MPN (n = 9; 10%) received pDLI (n = 62; 55%) or tDLI (n = 51; 45%). Median age at HSCT was 53 years (range, 19-72 years). Fifty-two pDLI recipients (84%) and 41 tDLI recipients (80%) were in morphological remission at HSCT. Donors were fully matched siblings in 51 cases

Table 2
DLI

DLI Characteristic	pDLI Cohort	tDLI Cohort
Infusion 1, n	62	51
Achievement of FDC, n (%)	11 (18)	
Time post-HSCT to DLI 1, days, median (range)	180 (87-1304)	277 (74-2801)
$CD3^+$ cell dose, cells/kg, median (range)	5×10^5 (1×10^5-5×10^6)	1×10^6 (5×10^5-1×10^7)
Time between infusions 1 and 2, days, median (range)	60 (20-149)	49 (16-189)
Infusion 2, n	39	34
Achievement of FDC, n (%)	13 (21)	
Time post-HSCT to DLI 2, days, median (range)	232 (139-482)	381 (90-1391)
$CD3^+$ cell dose, cells/kg, median (range)	1×10^6 (5×10^5-1×10^7)	5×10^6 (1×10^6-3.2×10^7)
Time between infusions 2 and 3, days, median (range)	46 (25-283)	53 (14-77)
Infusion 3, n	17	8
Achievement of FDC, n (%)	7 (11)	
Time post-HSCT to DLI 3, days, median (range)	363 (262-764)	597 (234-1468)
$CD3^+$ cell dose, cells/kg, median (range)	5×10^6 (4.5×10^6-1×10^7)	7.5×10^6 (5×10^6-5×10^7)
Time between infusions 3 and 4, days, median (range)	50 (30-125)	51 (34-152)
Infusions 4-8, n*	8	4
Achievement of FDC, n (%)	4 (6)	
Time post-HSCT to DLI 4, days, median (range)	404 (326-494)	403 (371-1535)
Total $CD3^+$ cell dose (doses 4-8), cells/kg, median (range)†	6.4×10^7 (1.65×10^7-1.66×10^8)	7.85×10^7 (1.65×10^7-1.43×10^8)

* The number (and percentage) of patients in the pDLI cohort who achieved FDC after 1, 2, 3, or 4-8 infusions (total, n = 35; 56%).
† Maximum number of doses given was 7 in the pDLI cohort and 8 in the tDLI cohort.

(45%) or matched unrelated donors in 62 cases (55%, of whom 41 [66%] were fully HLA matched at HLA-A, -B, -C, -DRB1, and -DQ; 21 [34%] were 1 or 2 antigen mismatched) There were no significant differences between the 2 cohorts with respect to disease or transplant characteristics (Table 1).

pDLI Administration and Chimerism Responses

Sixty-one patients received the first dose of pDLI at a median of 176 days (range, 87-461 days); a single patient received pDLI for a late development of MDC at 1304 days (Table 2). Median times from HSCT to subsequent doses were 232 days (range, 139-482 days) for dose 2, 363 days (range, 262-764 days) for dose 3, and 404 days (range, 326-494 days) for dose 4 (Table 2). Four patients received a fifth dose of pDLI at a median of 538 days (range, 367-776 days) post-HSCT, and 1 patient received a total of 7 doses.

pDLI was started at 5×10^5 $CD3^+$ cells/kg in 46 patients (74%); a lower starting dose of 1×10^5 $CD3^+$ cells/kg was given in 3 patients with recent grade II GVHD. The starting dose was 1×10^6 $CD3^+$ cells/kg (n = 10) or 5×10^6 $CD3^+$ cells/kg (n = 3), owing to very low chimerism (0% donor CD3) or poor risk features for HSCT (eg, persistent disease, adverse cytogenetics). Cyclosporine had been stopped by a median of 78 days post-HSCT (range, 39-229 days; treatment extended beyond 100 days in 1 patient with suspected, but subsequently unconfirmed, liver GVHD). All patients were off immunosuppression at the time of pDLI initiation; the median interval from cessation of cyclosporine to the first dose of pDLI was 70 days (range, 25-193 days).

The median donor CD3% in pDLI recipients before infusion was 23% (range, 0-77%). Median donor CD15 and unfractionated peripheral blood chimerism were 100% (range, 43%-100%) and 89% (range, 24%-100%), respectively. Chimerism response was evaluable in 56 patients; chimerism datasets were incomplete in 6 patients. Forty-two of these patients (67%) achieved sustained FDC (n = 35; 56%) or stable MDC (n = 7; 11%). Mean CD3% achieved was 76% (95% confidence interval [CI], 69%-83%) in the patients attaining stable MDC. Sixteen of these 42 responders (38%) received a single dose of pDLI; 14 (33%) received 2 doses (median total dose, 1.5×10^6 $CD3^+$ cells/kg; range, 1.1×10^6-1.5×10^7 $CD3^+$ cells/kg). Eight of them (19%) received 3 doses (median total dose, 6.5×10^6 $CD3^+$ cells/kg; range, 6.0×10^6-1.6×10^7 $CD3^+$ cells/kg) and 4 (10%) were given 4-7 doses (median total dose, 6.15×10^7 $CD3^+$ cells/kg; range, 1.65×10^7-1.64×10^8 $CD3^+$ cells/kg).

Fourteen of these 56 patients relapsed, at a median of 128 days (range, 13-394 days) after first pDLI. Eight of these patients showed no change in CD3%, whereas the other 6 had a maximum increase in donor CD3% of 20%-30%. The starting dose was 5×10^5 $CD3^+$ cells/kg in 11 of these patients; 3 patients had a higher starting dose (2 with 1×10^6 $CD3^+$ cells/kg and 1 with 5×10^6 $CD3^+$ cells/kg). Relapse occurred after 1-2 doses (n = 10; median total dose, 1.25×10^6, range, 5×10^5-1.5×10^7 $CD3^+$ cells/kg) or 3-5 doses (n = 4; median total dose, 6.4×10^7, range 1.15×10^7-1.66×10^8 $CD3^+$ cells/kg). Four of the 14 patients died, 5 underwent a second HSCT (median disease-free survival after second HSCT, 949 days; range, 136-2359 days), and 4 had stable disease at censor date (median OS post-pDLI, 987 days; range, 415-1542 days). The remaining patient received reinduction chemotherapy, consolidated with further DLI.

Absolute donor CD3% before pDLI was not significantly different between this group of 14 patients and those who attained sustained FDC or stable MDC ($P = .73$). In addition, there were no significant differences in the unfractionated peripheral blood ($P = .80$) or CD15 ($P = .80$) donor percentages between these 2 groups. No differences were found with respect to disease, transplantation (conditioning or donor type), or DLI variables (cumulative or starting dose and number of doses) (data not shown). Six patients had a total absence of detectable donor $CD3^+$ cell lineage; 3 of these patients showed no response to pDLI and relapsed, whereas the other 3 patients achieved FDC. Patients who attained sustained FDC or stable MDC post-pDLI had a significantly superior 5-year OS after pDLI of 91%, compared with 62% in the 14 patients who demonstrated no sustained improvement in chimerism after pDLI ($P < .01$).

Preemptive DLI for Low or Falling Chimerism Maintains High Remission Rates

The median follow-up after first pDLI was 1428 days (range, 75-3625 days). Five-year OS and EFS in 62 recipients

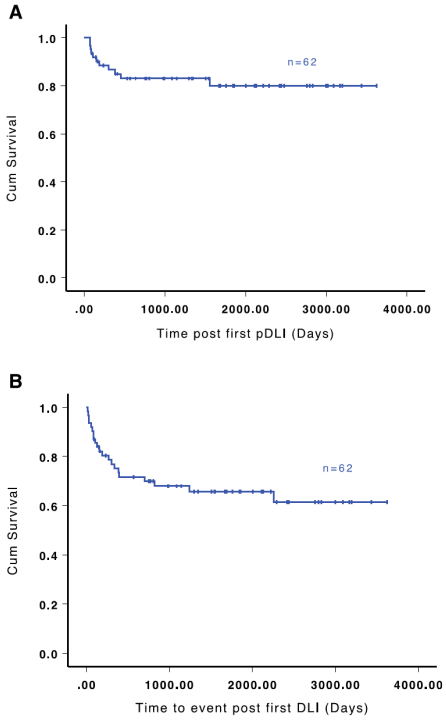

Figure 1. (A) Estimated OS for the pDLI cohort. OS calculated by the method of Kaplan-Meier demonstrated 80% 5-year survival after first pDLI. (B) Estimated EFS for the pDLI cohort. EFS calculated by the method of Kaplan-Meier demonstrated 65% 5-year survival after first pDLI.

of pDLI were 80% and 65%, respectively (Figure 1). No patient factor, disease variable (eg, primary diagnosis, cytogenetic risk group), or transplantation characteristic had a significant impact on OS or EFS (data not shown). Only 3 patients who achieved sustained FDC (n = 2) or stable MDC (n = 1) experienced subsequent disease relapse, at a median of 1240 days after first pDLI (range, 335-2257 days). Two of these 3 patients were salvaged with further DLI after remission reinduction. In total, 10 of 62 patients died, owing to disease relapse (n = 7), GVHD (n = 2), or sepsis (n = 1).

Thirty-two patients (52%) received pDLI within 6 months post-HSCT; the incidence of GVHD was only moderate, at 31% (n = 19) (Table 3). The median time to GVHD after first pDLI was 68 days (range, 34-398 days); a patient who received a total of 7 doses developed GVHD at 398 days after the first dose of pDLI. Four patients (6%) developed grade II-IV aGVHD, 3 (5%) developed limited GVHD, and 12 (19%) developed extensive cGVHD. Thirteen of the 19 patients (68%) who developed GVHD attained FDC, and 1 patient (5%) achieved stable MDC; logistic regression analysis showed that a chimerism response to pDLI was significantly associated with the development of GVHD ($P = .026$). No other variables, such as disease or transplantation characteristics (including donor type and stem cell source) or cumulative pDLI dose, were associated with the development of GVHD post-pDLI (data not shown).

Table 3
DLI-Related Toxicity and Causes of Death

Outcome	pDLI, n	tDLI, n
Death due to		
Relapse	7	23*
GVHD	2	1
Sepsis	1	2
Multiorgan failure, cause unknown	0	1
GVHD post-DLI		
Acute GVHD (total)	4	3
Grade II skin	1	2
Grade III-IV skin/gut/liver	3	1
Limited chronic GVHD (total)	3	1
Liver	2	0
Skin/oral	1	1
Extensive chronic GVHD (total)	12	17
Skin/liver/gut	10	12
Sclerodermatous chronic GVHD	2	5
GVHD therapy		
Topical only	2	2
Up to 2 systemic agents	7	10
Three systemic agents	4	8
Refractory GVHD requiring anti-TNF antibody	2	0
Data unavailable	4	1
Other complications of GVHD or its therapy		
Infection (including cytomegalovirus reactivation)	2	1
Steroid side effects (diabetes, avascular necrosis)	1	3
Surgical intervention for complications of gut GVHD†	1	0

* Includes deaths resulting from failure to respond to tDLI or subsequent relapse after response to tDLI.
† One cacostomy owing to perforated viscus.

Salvage Therapy Incorporating tDLI May Rescue a Proportion of Patients with Recurrent Disease Post-HSCT

Median time to relapse was 272.5 days (range, 62-2654 days). Median follow-up after first tDLI was 274 days (range, 23-3337 days). Estimated 5-year OS after tDLI was 40% (median, 705 days; 95% CI, 102-1308 days). Patients who relapsed before 6 months post-HSCT had a significantly inferior 5-year OS after tDLI (11% versus 51% for those who relapsed beyond 6 months; $P = .008$) (Figure 2) and a greater frequency of donor CD3% of <50% at relapse (60% versus 6% in those with later relapse; $P < .001$). Estimated 5-year OS after tDLI was 65% for patients with good-risk cytogenetics, 33% for those with intermediate-risk cytogenetics, and 0% for those with poor-risk cytogenetics ($P < .01$, good versus poor and intermediate versus poor) (Figure 3).

Cytogenetic data at relapse were available in 39 patients and included normal karyotype in 21, recurrence of original cytogenetic abnormality in 12, and new clonal changes (part of a complex karyotype in all) in 6. However, relapse karyotype had no impact on post-tDLI OS ($P = .39$). The presence of >5% blasts in the bone marrow before tDLI was also significantly associated with an inferior 5-year OS (24% versus 53% for patients in morphological complete remission (CR) before tDLI; $P < .01$).

Immunosuppression had been tapered and stopped by a median of 77 days (range, 40-485 days) after HSCT in this cohort. Nine patients (18%) were receiving immunosuppressive therapy at the time of relapse. Three patients relapsed while on immunosuppressive therapy to treat GVHD (beyond 100 days post-HSCT). Six other cases had relapsed while on immunosuppression in the early

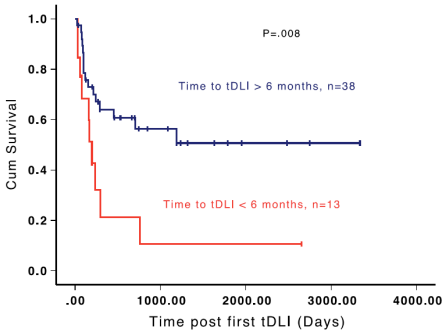

Figure 2. Estimated OS for the tDLI cohort, stratified by time to first DLI post-HSCT. Patients requiring tDLI at >6 months post-HSCT had a significantly superior OS after first tDLI compared with patients requiring therapy before 6 months.

post-HSCT period. No other disease- or HSCT-related variables affected OS post-tDLI (data not shown).

Thirty-five of the 51 patients who received tDLI also received chemotherapy (28 intensive, 7 low-dose; Table S3). Twenty-three patients achieved morphological CR post-chemotherapy. Eighteen remained in remission after tDLI; however, 8 patients relapsed later, and another 2 died (1 of GVHD and 1 of multiorgan failure). Eight patients were alive and disease-free at the time of censoring, with a median post-tDLI OS of 1711 days (292-2749 days).

Five patients who achieved CR post-chemotherapy relapsed during tDLI treatment. Another 12 patients showed no improvement in disease burden after chemotherapy and tDLI. Of these 17 patients, 4 were alive and receiving further chemotherapy at the time of censoring.

Sixteen patients received tDLI alone. All 4 patients who responded had cytogenetic relapse only. One patient died of sepsis after attainment of CR; 3 were alive at the time of censoring, with a median post-tDLI OS of 976 days (range, 464-3337 days). No difference in post-tDLI OS was seen between those patients who received both chemotherapy and tDLI and those who received tDLI alone ($P = .1$).

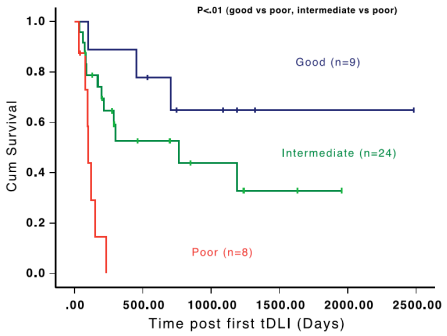

Figure 3. Estimated OS for the tDLI cohort, stratified by cytogenetic risk group at diagnosis [22,23]. OS at 5 years post-tDLI was estimated at 65% for good-risk patients, 33% for intermediate-risk patients, and 0% for poor-risk patients.

The starting dose of tDLI was 5×10^5 $CD3^+$ cells/kg in 18 patients (35%), 1×10^6 $CD3^+$ cells/kg in 13 patients (26%), 5×10^6 $CD3^+$ cells/kg in 9 patients (18%), and 1×10^7 $CD3^+$ cells/kg in 11 patients (22%), with subsequent dose escalation (Table 2). Twenty-two patients in remission after tDLI (18 after chemotherapy and tDLI and 4 after tDLI alone) received a median of 2 doses (range, 1-8), with a median total dose of 8.25×10^6 $CD3^+$ cells/kg (range, 5×10^5-1.43×10^8 $CD3^+$ cells/kg). Twenty-nine nonresponders (no response or disease progression during tDLI) received a median of 2 doses (range 1-4); median total dose was 9.5×10^6 $CD3^+$ cells/kg (range, 5×10^5-6×10^7 $CD3^+$ cells/kg). There was no significant difference in the cumulative dose of tDLI between responders and nonresponders (data not shown).

GVHD occurred in 21 patients (41%) after tDLI, including 3 (6%) with aGVHD and 18 (35%) with cGVHD, which was extensive in 17 (33%) (Table 3). Median time to the development of GVHD after first tDLI was 84 days (range, 19-323 days). GVHD occurred more frequently in those who experienced remission after tDLI, affecting 60% of responders versus 29% of nonresponders ($P = .03$), but had no impact on post-tDLI OS (data not shown). There was no significant difference in the mean cumulative dose of tDLI between patients who developed extensive cGVHD and those who developed only limited or no cGVHD ($P = .90$). Grade IV gut aGVHD accounted for 1 death. Relapse/refractory disease accounted for the majority of the deaths (86%; 23 of 27) in the tDLI cohort.

DISCUSSION

The advent of RIC has made HSCT feasible in older patients with AML and MDS [3-5], as illustrated by our cohort's median age of 54 years. TCD aims to facilitate engraftment in RIC HSCT and to reduce the incidence of GVHD in high-risk older patients [11,27]. However, RIC [4,5,28] and TCD [8,9] have been implicated in the persistent risk of relapse post-HSCT, through a reduced GVL effect. TCD is frequently associated with prolonged mixed donor chimerism, most clearly described after alemtuzumab administration [29,30]. An increased risk of relapse has been reported in patients with MDC or progressively decreasing donor chimerism [15,31], suggesting a role for pDLI in improving chimerism and boosting the GVL effect. This was recently demonstrated after TCD RIC HSCT for lymphoma, where pDLI effectively converted MDC to FDC and reduced the risk of relapse [32,33].

The aim of the present analysis was to review outcomes after pDLI and tDLI in a single-center cohort of patients with AML and MDS after a RIC protocol including in vivo T cell depletion with alemtuzumab or ATG. We have demonstrated that pDLI given to revert low or falling donor CD3 chimerism after TCD RIC HSCT (predominantly incorporating alemtuzumab) can effectively prevent relapse. Only 3 patients with sustained FDC or stable MDC after pDLI subsequently relapsed (at a minimum of 335 days after pDLI). This suggests that pDLI provides effective GVL activity against AML/MDS even in patients at high risk for disease recurrence. In contrast, relapse was observed in all patients who did not show a sustained improvement in donor chimerism after pDLI.

More than one-half of our cohort of older patients received pDLI before 6 months post-HSCT. Despite early treatment with pDLI, rates of GVHD were only moderate in the pDLI cohort; 19 patients developed GVHD, 12 with extensive cGVHD. Only 2 deaths were attributed to GVHD.

Furthermore, infectious complications (including cytomegalovirus reactivation) related to GVHD or its therapy were infrequent (2 patients; Table 3). Our results contrast with a 2002 registry study of DLI administration after TCD RIC HSCT, suggesting high rates of GVHD (>50%) after treatment before 6 months [13]. In this study of 81 patients with lymphoid and myeloid malignancies receiving DLI, 18 were treated for MDC. Most patients had undergone HSCT with persistent disease, not all had received alemtuzumab, and the dosage varied among regimens (100 mg versus 50 mg) [13]. Although there has been a report of DLI administration in some cases even before 100 days after myeloablative and RIC HSCT [34], the present study is the first to report results of early, escalating-dose pDLI in a uniform cohort of TCD RIC HSCT recipients with AML and MDS.

In the setting of tDLI, superior 5-year OS was seen in patients requiring tDLI for relapse more than 6 months after TCD RIC HSCT and those with a pre-tDLI bone marrow blast percentage <5%. Interestingly, karyotype at diagnosis also influenced 5-year OS. The majority of patients in our cohort had received chemotherapy in combination with tDLI, making it difficult to separate out the contributions of each modality to our results. When tDLI was given alone, it was efficacious only in those with low tumor burden (cytogenetic relapse only). Our findings confirm previous results of tDLI after myeloablative HSCT from Schmid et al. [20], who reported improved OS at 2 years for patients who relapsed beyond 5 months or in morphological remission before tDLI.

Delayed immune reconstitution has been reported after TCD HSCT, particularly when alemtuzumab is used. This delay is associated with infection, viral reactivation [11,35], and a negative impact on GVL activity [8,9]. Indeed, the statistically significant association of GVHD with chimerism response to pDLI and attainment of remission in our tDLI cohort supports the provision of an active alloimmune response by DLI in responding patients. An earlier study from our center found a trend toward fewer relapses of AML/MDS after pDLI in patients with MDC compared with patients with spontaneous FDC or stable MDC who had not received DLI [17]. The beneficial effects specifically of pDLI may reflect correction of deficiencies in the immune cell repertoire after TCD HSCT. We have previously demonstrated that although early lymphocyte recovery was robust in patients after fludarabine, busulfan and alemtuzum HSCT for AML/MDS who subsequently relapsed [12], there were marked deficiencies in the frequency of $CD4^+$ T cells and naïve T cells in both the $CD4^+$ and $CD8^+$ cell populations for at least 6 months after HSCT [36].

After HLA-matched HSCT, T cells capable of recognizing minor histocompatibility antigen (mHag) disparities may contribute to GVL activity; by definition, such donor-derived T cells will be naïve [37,38]. mHag-specific T cell clones capable of lytic activity against leukemia cells have been isolated from patients after DLI [39,40]. Thus, a possible mechanism for the GVL activity of DLI may be the provision of naïve donor-derived T cells, which increases the T cell repertoire capable of tumor cytotoxicity; this remains an exciting area of future research.

In conclusion, our data support the utility of TCD RIC HSCT as an immune platform for treating AML and MDS, in which the judicious early use of pDLI can safely promote durable remission. Therapeutic DLI may be combined with chemotherapy to salvage a proportion of patients with recurrent disease, particularly those who relapse beyond 6 months post-HSCT. Prospective studies are needed to more clearly address the efficacy of this intervention by comparing outcomes in patients after DLI and patients not exposed to this therapy.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2012.12.013.

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3.2.1 Supplementary data

Table S1. Details of conditioning regimens

FBC (n=99)	Fludarabine 30 mg/m^2 iv D-8 to D-4
	CAMPATH (Alemtuzumab) 20mg iv D-8 to D-4
	Busulphan 3.2 mg/kg iv or 4mg/kg po D-3 to D-2*
FBATG (n=12)	Fludarabine 30mg/m^2 iv D-9 to D-5
	Busulphan 3.2 mg/kg iv D-4 to D-3
	ATG 6mg/kg (total dose) iv D-4 to D-1
FLAMSA (n=2)	Fludarabine 30mg/m^2 iv D-12 to D-9
	Cytarabine 2g/m^2 iv D-12 to D-9
	Amsacrine 100mg/m2 iv D-12 to D-9
	Busulphan 3.2mg/kg iv D-5 to D-4
	ATG 2.5mg/kg iv D-4 to D-2

* 19 patients received total of 4 doses of busulphan D-5 to D-2
ATG, Anti-thymocyte globulin

Table S2. DLI dose escalation schedule.

DLI dose: total CD3+ cell dose/kg	$5x10^5$, $1x10^6$, $5x10^6$, $1x10^7$, $5x10^7$, $1x10^8$

Table S3. tDLI cohort: relapse and treatment characteristics

Characteristic	No of patients (total=51)
Cytogenetics at relapse	
Normal	21
Recurrence of original abnormality	12
Recurrence of original + new clonal abnormality	6
Cytogenetics failed/missing	12
Blast percentage pre tDLI	
<5%	32
>5%	14
5-10%	12
>10%	2
missing	5
Intensive chemotherapy (total)	28
FLAG/FLAG-Ida	6
High Dose Cytarabine	1
DA	3
ADE	2
Melphalan	1
Gemtuzumab-ozogamicin	5
Non-intensive chemotherapy (total)	7
Low dose cytarabine	6
5-Azacytidine	1

3.3 Discussion

This article was published at the same time as a report by Liga et al. presenting data from 15 patients after myeloablative HSCT incorporating alemtuzumab who were given DLI pre-emptively for mixed donor chimerism, or prophylactically due to perceived high risk of recurrent acute leukaemia[279]. Using low doses of DLI (median cumulative cell dose $2x10^6$ CD3+ cells/kg) delivered at a median of 162 days post-HSCT, a high relapse-free survival (72% +/-12% at 2 years) in 8 recipients of pDLI for mixed chimerism was observed[279]. Six (75%)of the mixed chimeric patients converted to complete donor chimerism following DLI. However, incidence of GvHD was 50% in these patients and overall, a DLI-related mortality (principally due to GvHD) of 27% was described[279]. Whilst the favourable response rates are encouraging, it should be noted that the threshold for commencement of pDLI for mixed chimerism was as low as 5% recipient cells i.e. 95% donor cells, the median being a recipient unfractionated chimerism in the peripheral blood cells of 7.5% (range 5-15%)[279]. Also of note, doses of alemtuzumab used in these patients following a myeloablative protocol ranged from 10-20mg, which is substantially lower than the 100mg used in our patients. In combination, it is likely that such differences account for the higher incidence and severity of post-DLI GvHD reported in this study.

These issues were highlighted in an accompanying editorial published in Biology of Blood and Marrow Transplantation, 2013, 19(4): 507-508, [280] which commented on our findings and those of Liga et al. The encouraging disease-free survival reported by both studies in patients experiencing improved chimerism following pDLI, supporting provision of effective GvL activity by pDLI in responders was highlighted. The multitude of variables that impact upon the risk of GvHD following DLI was discussed, with emphasis on use (and type) of TCD in the initial conditioning regimen, CD3+ T-cell dose administered in DLI, timing post-HSCT and degree of HLA-matching between donor and recipients[280]. It is evident that prospective studies, in the context of uniform patient cohorts with clearly identified indications and dose scheduling of pDLI, incorporating patients not requiring pDLI as a control arm, are required to increase knowledge of the risks and benefits of this therapy.

Our data support the potential for DLI to promote GvL activity without inducing unacceptable rates of severe GvHD. In the context of relapsed disease, tDLI was most effective in the setting of low disease burden (complete morphological remission or recurrent cytogenetic disease only) in patients relapsing later than 6 months post-HSCT. The 5-year relapse/progression rate of 69% (95% CI, 54%-81%) in recipients of tDLI highlights the need to boost the induction of sustained GvL responses in these patients. One means to achieve this may be by combining tDLI with active immunotherapeutic strategies, such as the AML Cell Vaccine.

Chapter 4 Combined Adjuvants for Synergistic Activation of Cell-Mediated Immunity (CASAC) combine with WT1 peptide vaccination to induce WT1-specific T-cell responses

4.1 Introduction

The data presented in Chapter 1 outlined the rationale for immunotherapeutic targeting of the WT1 protein in tumours that frequently over-express this transcription factor, such as AML. Whilst early phase clinical studies have shown that it is possible to induce WT1-specific immune responses in AML and MDS patients, the short-lived T-cell expansions of low magnitude resulting from vaccination suggest a requirement for additional factors to promote durable and potent cytotoxic T-cell activity[198-201]. A major limitation in cancer vaccination trials has been the absence of clinically safe, effective adjuvants for the promotion of cell-mediated immunity. Advances in our understanding of the importance of TLR agonists in licensing DCs to provide essential signals for activation of cognate naive T-cells has offered the possibility of introducing more powerful adjuvants into the clinical setting[227,234]. Previous work from our group highlighted the ability of selected adjuvants to synergise to induce large expansions of antigen-specific T-cells following repeated vaccination in a murine model. This was effective in the context not only of a xenoantigen but also a self-antigen, TRP-2, which is overexpressed by the B16 melanoma cell line. Specifically, CASAC/TRP-2 vaccinations were able to prolong the survival of B16 melanoma-bearing C57BL/6 mice[238].

Given these observations and the immunotherapeutic potential of targeting WT1, the ability of CASAC to enhance WT1-specific immunity was assessed in the following studies. C57BL/6 mice were immunised with WT1-RMF, a well-defined WT1 epitope in the context of both HLA-A*02:01 in humans and H-2D^{b} in mice[159,173,179] and induction of WT1-specific T-cell responses was examined. Other factors that may combine with CASAC to increase the potency of WT1-specific T-cell responses or extend the applicability of WT1 peptide vaccination to all patients, irrespective of HLA-type, were also evaluated. A heteroclitic modification of the WT1-RMF epitope (WT1-YMF), reported to be more effective at inducing immune responses in CTLs that cross-react with native WT1-RMF in the context of human HLA A*02:01

presentation[190,201,203], was compared with wild-type WT1-RMF in CASAC vaccination studies in H-2^b-expressing C57BL/6 mice.

Additionally, a promising approach to vaccination studies, namely use of an overlapping peptide pool for immunisations, was studied. Such a strategy has shown efficacy in the therapy of cervical cancer and human papilloma virus (HPV) positive vulval intraepithelial neoplasia in Phase II clinical trials but has not been previously explored in the context of vaccinations targeting WT1[216,217]. A pool of overlapping WT1 peptides is available from Miltenyi Biotec Ltd (WT1 PepTivator®). This comprises a cocktail of lyophilized peptides, mainly 15-mer sequences with 11 amino acid overlap, covering the complete sequence of the human WT1 protein. It has been created principally for the purpose of generating WT1-specific CTLs *in vitro* for subsequent adoptive immunotherapy against WT1-expressing malignancies. A Good Manufacturing Practice (GMP) Grade version of the WT1 PepTivator® is also available and therefore this product could be translated to the clinical setting with ease.

The following studies demonstrate the feasibility of CASAC vaccinations targeting WT1 to induce WT1-specific T-cell expansions demonstrating functional efficacy in the *in vivo* setting. Strategies to optimise and extend this approach that could translate WT1 peptide vaccination to the broader patient population are also explored.

4.2 Results

4.2.1 Feasibility and specificity of WT1 peptide and CASAC vaccination

Previous studies by Wells et al had demonstrated that immunising C57BL/6 mice against the ovalbumin-derived Class I peptide OVA-SIINF in combination with either a Class II peptide or an agonistic anti-CD40 antibody, IFNγ and the TLR agonists CpG and poly I:C, all admixed with an emulsion containing 0.4% v/v Tween 80, 4.4% v/v squalene in PBS, induced OVA-SIINF specific CD8+ T-cell expansion. Vaccinations administered on days 1 and 10 using these components resulted in detection of a population of OVA-SIINF-specific CD8+ T-cells using pentamer-based quantification of antigen-specific T-cells on day 20. This population accounted for approximately 25-30% of all circulating CD8+ T-cells[238].

A similar schedule was therefore adopted to determine whether vaccinations against WT1-RMF combined with CASAC could result in the induction of a WT1-RMF specific immune response. The heteroclitic modification of WT1-RMF, WT1-YMF, was predicted to show superior binding to H-2D^b than WT1-RMF using the SYFPEITHI prediction tool and the IEDB database (Table 4-1). Groups of mice undergoing vaccinations incorporating the OVA-SIINF peptide and CASAC (as described above) were used as positive controls in these studies to confirm biologic activity of the CASAC components.

Table 4-1 Binding affinity of WT1-RMF and WT1-YMF to H-2D^b

Epitope	SYFPEITHI score§	IC_{50} (nM, ANN method)¶
WT1-RMF	24	81
WT1-YMF	25	24

§SYFPEITH scores performed using the SYFPEITHI database[180]
¶ Artificial Neural Network method used by IEDB (www.iedb.org) to report pMHC binding affinities[281,282]
The lower the IC_{50} value, the higher the binding affinity of the peptide for the class I molecule.

4.2.1.1 Vaccinations combining CASAC and WT1-RMF induce expansion of WT1-RMF specific T-cells and WT1-RMF specific target cell lysis *in vivo*

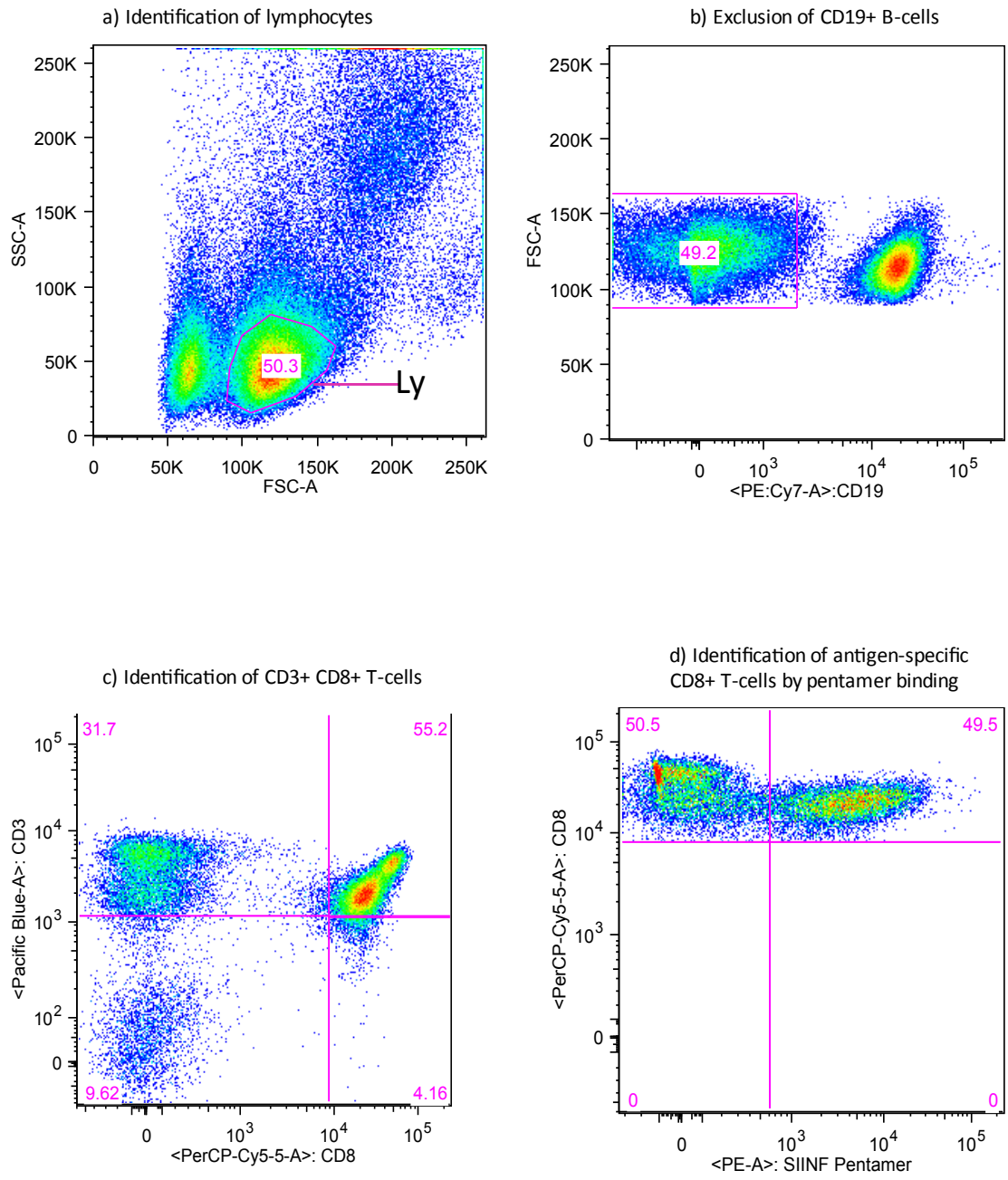
Groups of mice (5 per group) were immunised with OVA-SIINF, WT1-RMF or its heteroclitic modification, WT1-YMF. Either no Class II peptide, the Class II ovalbumin derived peptide ISQAVHAAHAEINEAGR$_{323-339}$ (OVA-ISQ) or the PAn-HLA-DR-Epitope (PADRE) were combined with Class I peptide and CASAC components (CpG, poly I:C and IFNγ, mixed 50:50 with emulsion as above). One hundred μg of each peptide were administered per mouse, per injection, as described previously by Wells et al and CASAC components were also used in quantities established to be effective in the original publication[238]. OVA-ISQ had been used in the context of CASAC vaccinations whereas PADRE had not been assessed previously in CASAC studies. The PADRE 965.10 peptide (KSSAKXVAAWTLKAAA) is a synthetic Class II helper peptide designed to bind to a range of Class II molecules to activate CD4+ T-helper responses. Studies carried out during its initial development demonstrated this peptide's ability to effectively promote *in vivo* CD4+ T-cell responses in C57BL/6 mice and to bind to HLA Class II and stimulate human T-cell proliferation *in vitro*[266]. One rationale for investigating PADRE within CASAC was to determine whether it might be useful as a universal helper peptide that could allow comparisons between vaccinations with varying Class I peptides or CASAC components. Furthermore, if demonstrated to be effective as a helper peptide in pre-

clinical models, PADRE could easily be used as a helper peptide in studies of peptide vaccination with CASAC in humans, since it has been shown to be safe in clinical trials thus far[283,284].

After 2 rounds of vaccination, on day 20, mice were bled to assess for antigen-specific CD8+ T-cell responses using fluorescent pentamers containing the peptide against which the mouse had been immunized. Two types of negative controls were used: 1) unimmunized mice stained with the pentamers containing the immunizing peptide and 2) an irrelevant pentamer containing a peptide derived from the lymphochoriomeningitis virus (LCMV), to which mice housed in the pathogen-free environment should not have been exposed and therefore only low (background) frequency of LCMV-specific CD8+ T-cells would be expected. Figure 4-1 (a-d) illustrates the gating strategy used to determine the frequency of antigen-specific T-cells in a representative OVA-SIINF/ISQ immunized mouse by staining of murine PBMCs. This gating strategy was applied to PBMCs collected from all immunized and unimmunized mouse groups. The frequencies of antigen specific CD8+ T-cells detected in mice immunized against either OVA-SIINF or WT1-RMF after 2 rounds of vaccination are illustrated in Figures 4-2 and 4-3.

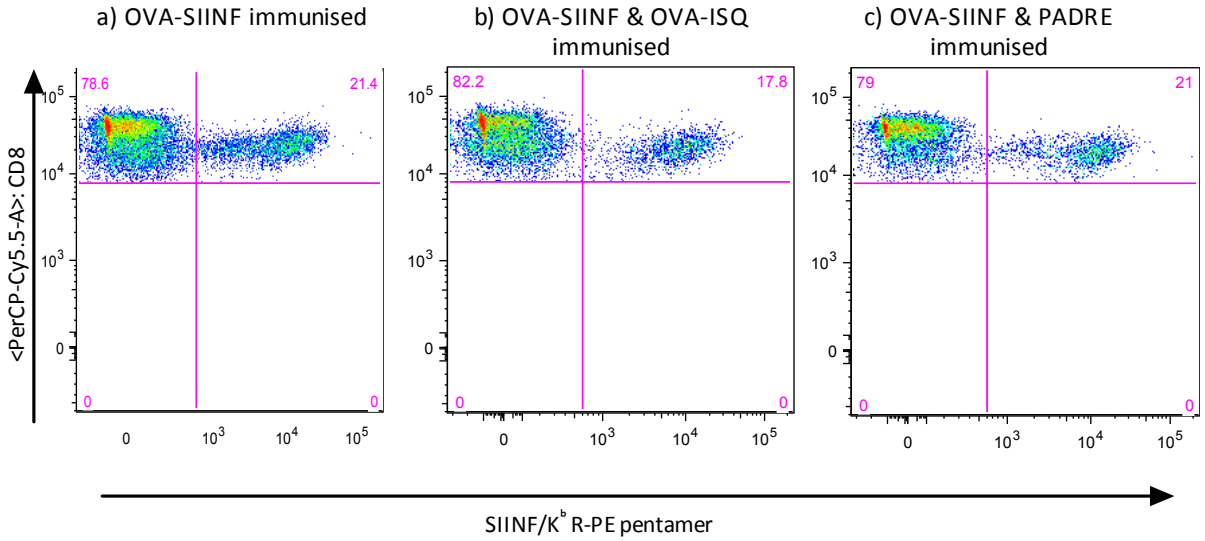
Mean frequencies of OVA-SIINF specific CD8+ T-cells following 2 rounds of vaccination ranged from 18-24% amongst the 3 groups with no significant differences between groups observed. These mean percentages are comparable with the results achieved in the original paper by Wells et al, suggesting that the experimental procedure recapitulated the published protocol[238]. Within each OVA-SIINF immunized group, there was variability in terms of the magnitude of OVA-SIINF specific T-cell expansion that was not specifically commented upon in the original publication but has been observed by others in our group (Gee Jun Tye, unpublished observations). The basis for this variability between individuals within a cohort of vaccinated mice is not at present understood.

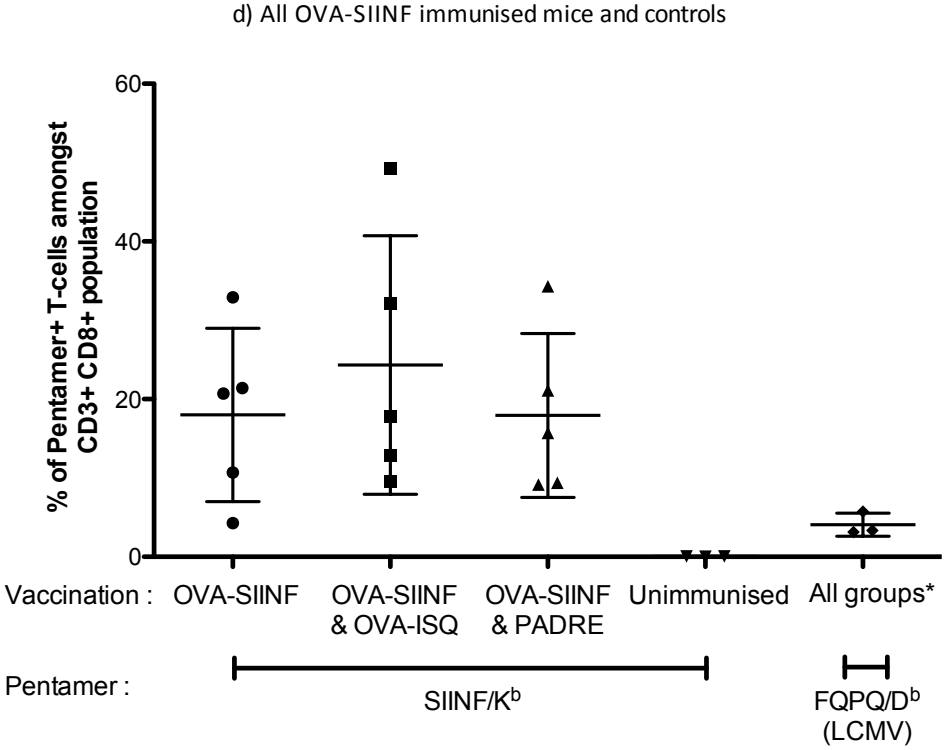
Figure 4-1 Gating strategy for pentamer studies.



Fluorescently labelled PBMCs from a representative OVA-SIINF/ISQ-immunised mouse following 2 vaccinations are shown. (a) Lymphocytes (Ly) were identified based on forward (FSC) and side (SSC) scatter properties. (b) A plot of FSC versus CD19 expression identified CD19+ B-cells and CD19- populations: a gate including all CD19- events was created. (c) The CD19- population was then analysed for expression of CD3 and CD8 to allow identification of CD3+ and CD8+ T-cells. (d) The CD3+ CD8+ population was then assessed for binding of the K^b/SIINF pentamer by plotting CD8 against K^b/SIINF.

Figure 4-2 Expansion of OVA-SIINF specific CD8+ T-cells following 2 rounds of vaccination with OVA-SIINF and CASAC in the presence or absence of a helper peptide.



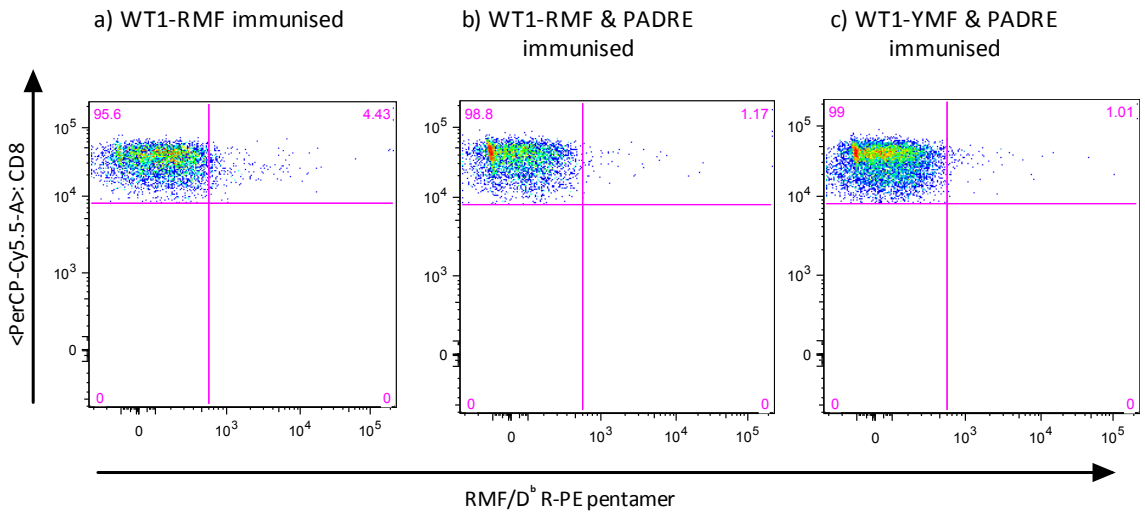


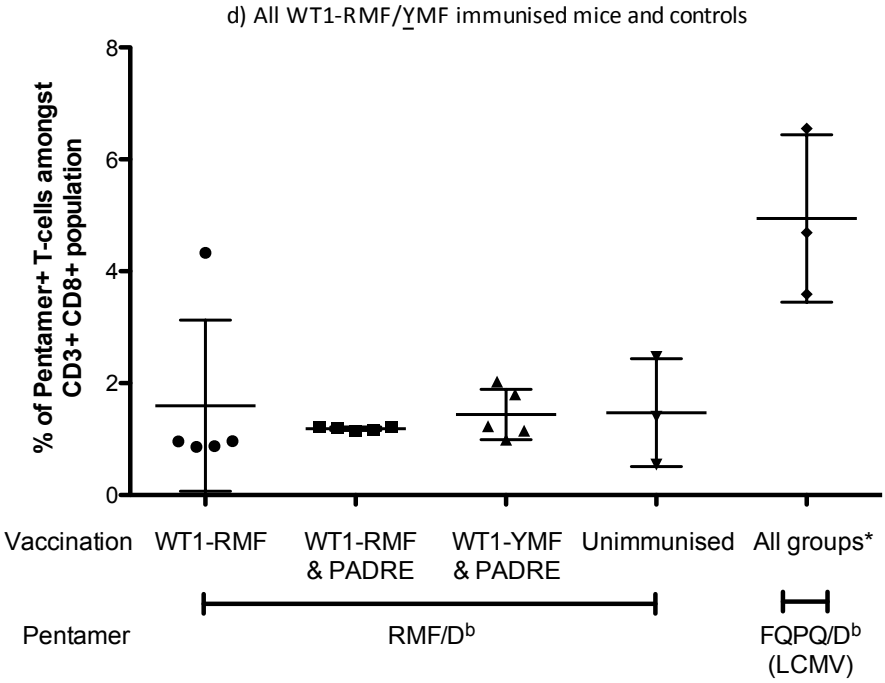
Groups of up to 5 mice were immunised with CASAC and using 100 μg OVA-SIINF alone or in combination with 100 μg OVA-ISQ or PADRE per mouse. Unimmunised mice served as controls. CASAC consisted of the following components per vaccination: CpG 1826 25μg, Poly I:C 50μg, IFNγ 1ng admixed in a 4.4% squalene emulsion (see Chapter 2 for further details). Pentamer staining was used to detect the frequency of CD8+ T-cells recognizing OVA-SIINF/K^b in the peripheral blood. All groups*: a sample from each immunised group was stained with an irrelevant pentamer containing the LCMV-derived peptide FQPQ, serving as a negative control. Mean and standard deviation (SD) for each group of mice is shown. No significant differences in the frequency of antigen-specific T-cells between any of the groups were detected. OVA, ovalbumin; SIINF, SIINFEKL; ISQ, ISQAVHAAHAEINEAGR; PADRE, Pan-HLA-DR Epitope; FQPQ, FQPQNGQFI; LCMV, Lymphochoriomeningitis virus; K^b, H-$2K^b$.

In the same experiment, mice immunised with CASAC in combination with WT1-RMF (+/- PADRE) or YMF + PADRE were also assessed after 2 rounds of vaccination for the development of WT1-RMF specific CD8+ T-cell expansion (Figure 4-3). Only one mouse of five in the WT1-RMF vaccinated group had pentamer staining 4.4% above the background for unimmunised mice. No pentamer staining above background was seen in any mouse following immunization with WT1-RMF & PADRE or WT1-YMF & PADRE. Given the requirement to break tolerance against a self-antigen in the vaccinations targeting WT1, contrasting with the immunogenicity of peptides deriving from the xenoantigen OVA, the absence of detectable WT1-RMF specific CD8+ T-cells following only 2 rounds of vaccination in all except one mouse was not entirely surprising.

To determine whether repeated exposure to WT1 peptides and CASAC could promote expansion of WT1-RMF specific CD8+ T-cells, vaccinations were continued in these mice for a further 2 rounds, given on days 24 and 36. The frequencies of antigen-specific T-cells were analysed by pentamer analyses on days 35 and 45. As a comparator, vaccinations were similarly continued in the OVA-SIINF exposed groups; results following 4 rounds of vaccination are presented in Figure 4-4a and b. High levels of background staining were observed for the LCMV peptide-containing pentamer FQPQ/D^b-R-PE in this experiment but were reduced in subsequent studies by centrifugation of pentamers for 10 minutes prior to use to avoid pipetting of aggregates into the wells.

Figure 4-3 Low frequencies of WT1-RMF specific CD8+ T-cells following 2 rounds of vaccination with WT1-RMF or the heteroclitic peptide WT1-YMF with CASAC, in the presence of absence of the helper peptide PADRE





In the same experiment described in Figure 4-2, groups of up to 5 mice were immunised using WT1-RMF alone or in combination with PADRE or WT1-YMF and PADRE. Unimmunised mice served as controls. Pentamer staining was used to detect the frequency of CD8+ T-cells recognizing WT1-RMF/K^b in the peripheral blood. A sample from each immunised group was stained with an irrelevant pentamer containing the LCMV-derived peptide FQPQ, serving as a negative control. Mean and SD for each group of mice is shown. No significant differences in the frequency of antigen-specific T-cells between any of the groups were detected. Please note the differences in y-axis scale between Figures 4-2 and 4-3. WT1, Wilms' Tumour protein; RMF, RMFPNAPYL; YMF, YMFPNAPYL (Y denotes substitution of tyrosine for arginine at position 1); PADRE, Pan-HLA-DR Epitope; FQPQ, FQPQNGQFI; LCMV, Lymphochoriomeningitis virus; K^b, H-2K^b.

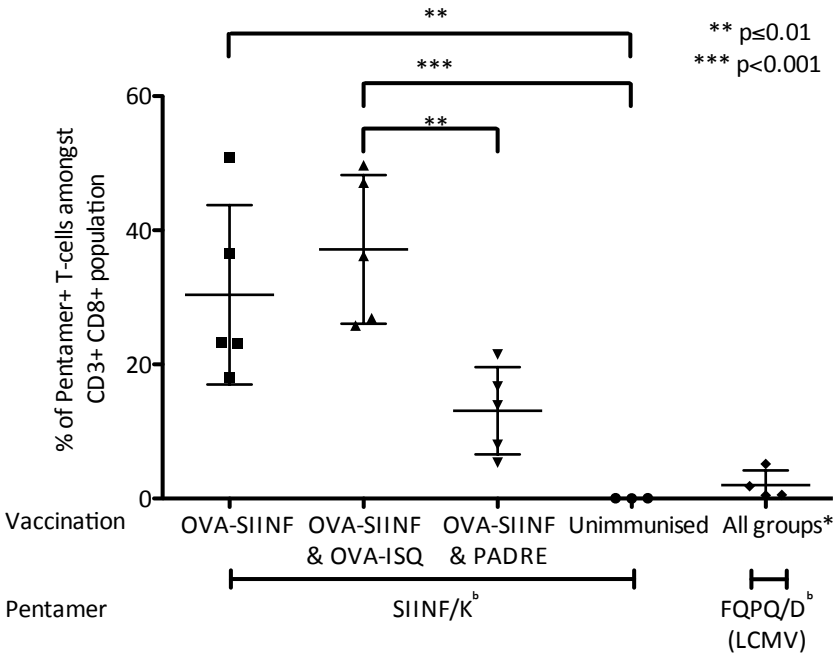
Following 4 rounds of vaccination, an increase in the mean frequencies of OVA-SIINF-specific CD8+ T-cells was detected for both the OVA-SIINF and OVA-SIINF & ISQ immunized groups (30.4% and 37% respectively, Figures 4-4a and b). The mean frequencies of OVA-SIINF-specific T-cells in these 2 groups were significantly higher than those observed in the unimmunized group ($p \leq 0.01$ for OVA-SIINF versus unimmunized and $p \leq 0.001$ for OVA-SIINF & ISQ), consistent with the findings of Wells et al that expansion of CD8+ T-cells may be enhanced where vaccinations include a Class II peptide[238]. However, the mean frequency of OVA-SIINF-specific CD8+ T-cells was significantly higher in the OVA-SIINF & ISQ-immunised mice than the OVA-SIINF&PADRE group (where the mean frequency was 13%), Figures 4-4a and b. These findings suggested that PADRE might not be as effective as the OVA-derived helper peptide OVA-ISQ in the context of vaccinating C57BL/6 mice.

After 4 rounds of vaccination, a rise in the frequency of WT1-RMF specific T-cells was observed in 2 mice within the group receiving WT1-RMF Class I peptide alone with CASAC, suggesting the induction of an antigen-specific immune response (Figure 4-4c). The remaining 3 mice did not show such an expansion, implying that while CASAC can combine with WT1-RMF to induce WT1-RMF specific T-cell expansion following 4 vaccinations, this response is not consistently observed in all subjects within one group. There is a suggestion that the 2 mice showing the highest responses after 3 vaccinations exhibited a reduction in the frequencies of WT1-RMF specific T-cells after the fourth vaccination (Figure 4-4d). In this particular experiment the mice were not tagged to allow identification of individual responses. This observation may represent a true waning in the frequency of WT1-RMF specific T-cells in the peripheral blood following 4 vaccinations. However, it could also reflect the difference in timings of phlebotomy following vaccinations (at 11 days post the third and 9 days post the fourth vaccination).

Similarly, two of the five mice in the WT1-RMF & PADRE and the WT1-YMF & PADRE immunized groups respectively showed increased WT1-RMF pentamer staining above background following 4 rounds of vaccination (Figure 4-4c). However, in both groups, the frequencies of WT1-RMF specific T-cells were lower than the 2 highest responses in the WT1-RMF immunized group. This suggests that addition of PADRE to WT1-RMF or use of WT1-YMF for induction of WT1-RMF specific responses is not beneficial and may even be detrimental. Due to variability of the response rates between groups, these differences were not shown to be statistically significant.

Figure 4-4 Induction of antigen-specific T-cells following 4 rounds of vaccination against OVA-SIINF or WT1-RMF with CASAC

a) Frequency of OVA-SIINF specific CD8+ T-cells after 4 rounds of vaccination



b) Frequency of OVA-SIINF specific CD8+ T-cells after 2, 3 & 4 rounds of vaccination

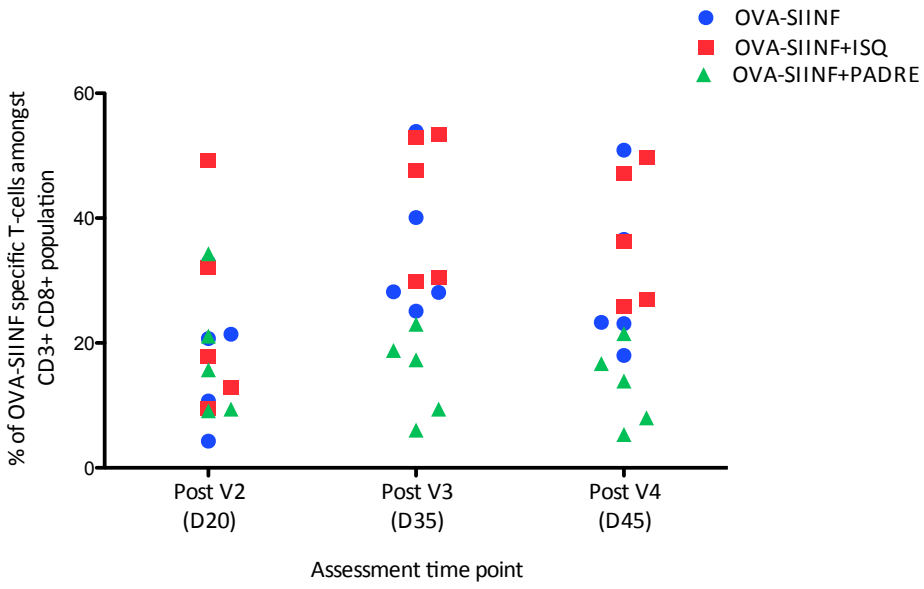
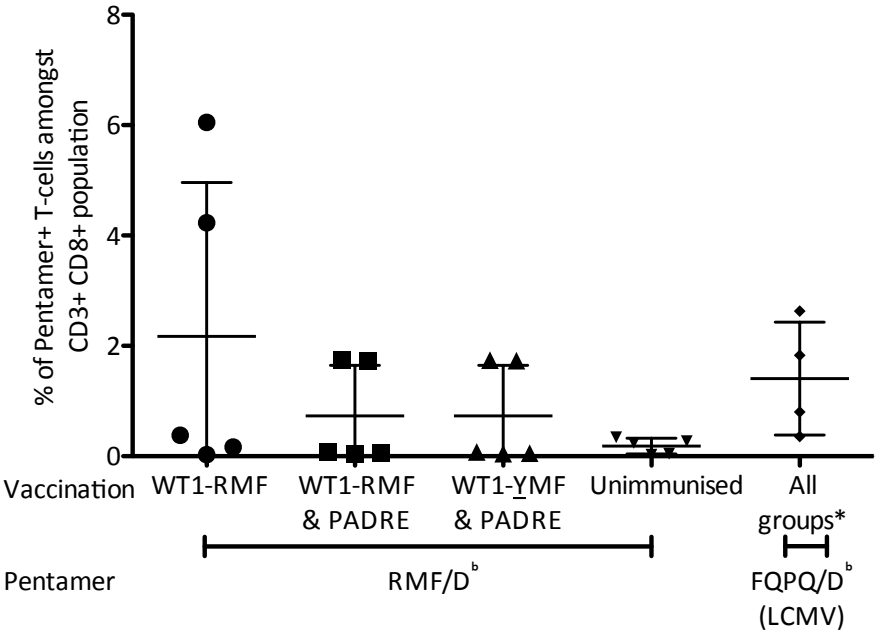


Figure 4-4(a) Following 4 rounds of vaccination, in the same groups of mice depicted in Figure 4-2, OVA-SIINF specific CD8+ T-cell frequencies (including group means and SD) were determined by pentamer analysis. Results of staining with the irrelevant LCMV-specific FQPQ/D^b pentamer in a representative sample from each group are also shown (All groups*). (b) Longitudinal representation of individual frequencies of OVA-SIINF specific CD8+ T-cells in each group after 2, 3 and 4 rounds of vaccination. No significant differences (by 2-way analysis of variance, ANOVA) were observed in the frequencies of antigen-specific CD8+ T-cells per group at the 3 different time points.

c) Frequency of WT1-RMF specific CD8+ T-cells after 4 rounds of vaccination



d) Frequency of WT1-RMF specific CD8+ T-cells after 2,3 & 4 rounds of vaccination

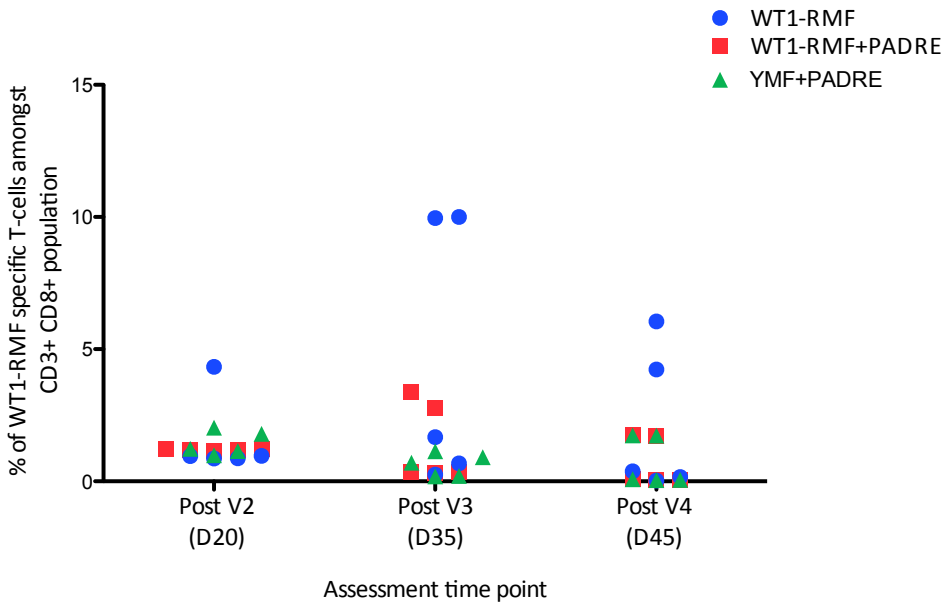


Figure 4-4 continued.
4-4 (c) Following 4 rounds of vaccination, in the same groups of mice depicted in Figure 4-3, WT1-RMF specific CD8+ T-cell frequencies (including group means and SD) were determined by pentamer analysis. Results of staining with the irrelevant LCMV-specific FQPQ/D^b pentamer in a representative sample from each group are also shown (All groups*). (d) Longitudinal representation of individual frequencies of WT1-RMF specific CD8+ T-cells in each group after 2, 3 and 4 rounds of vaccination. No significant differences (by 2-way analysis of variance, ANOVA) were observed in the frequencies of antigen-specific CD8+ T-cells per group at the 3 different time points.

***In vivo* cytolysis assay**

An *in vivo* cytotoxicity assay was carried out as a functional assessment of WT1-RMF-specific immune responses. The relative *in vivo* lysis of OVA-SIINF or WT1-RMF peptide-loaded syngeneic splenocytes in comparison to non-loaded splenocytes was assessed in OVA-SIINF or WT1-RMF/YMF immunized mice respectively. The fluorescent label, carboxyfluorescein diacetate, succinimidyl ester (CFSE) was used to differentially label target and non-target populations to give low or high intensities of fluorescence staining (Figure 4-5c). Unimmunized mice served as controls. The assay was carried out on day 48, 12 days following the fourth vaccination. Relative lysis of the 2 CFSE labeled populations in control and immunized mice allowed a measure of peptide-specific target cell lysis to be made for each mouse. The gating strategy is depicted in Figure 4-5. The results of the *in vivo* cytolysis assay in OVA-SIINF and WT1-RMF/YMF immunized mice are shown in Figure 4-6.

CFSE-labelled splenocytes ($1x10^7$), comprising a 50:50 mixture of CFSE "high" intensity staining, non-peptide loaded targets and CFSE "low" intensity staining, peptide-loaded targets were injected i.v. into each of the immunised and unimmunised mice. Eighteen hours later, spleens were harvested from each mouse and assessment of the relative proportions of the 2 CFSE-labelled populations used to determine the degree of antigen-specific lysis of target peptide-loaded cells. Mice immunised with OVA-SIINF were challenged with a mixture containing OVA-SIINF-loaded splenocytes; mice immunised with WT1-RMF or YMF were challenged with a mixture containing WT1-RMF-loaded splenocytes. Of the 5 control mice, 1 received a mixture of splenocytes containing OVA-SIINF-loaded targets whilst the remaining 4 received a mixture containing WT1-RMF-loaded targets. As an example, the calculation to determine percentage lysis of peptide-loaded target cells, based on the results shown in Figures 4-5 and 4-6, is outlined below:

Calculation of antigen-specific lysis of target cells in the *in vivo* cytolysis assay

Unimmunised control mouse challenged with WT1-RMF targets:

$$\frac{\text{Number of events in the CFSE "low" target population}}{\text{Number of events in the CFSE "high" non-target population}} = \frac{3716}{2986} = 1.24$$

Averaged ratios from 4 unimmunised mice = [1.24+1.28+1.29+1.29]/4 = 1.28

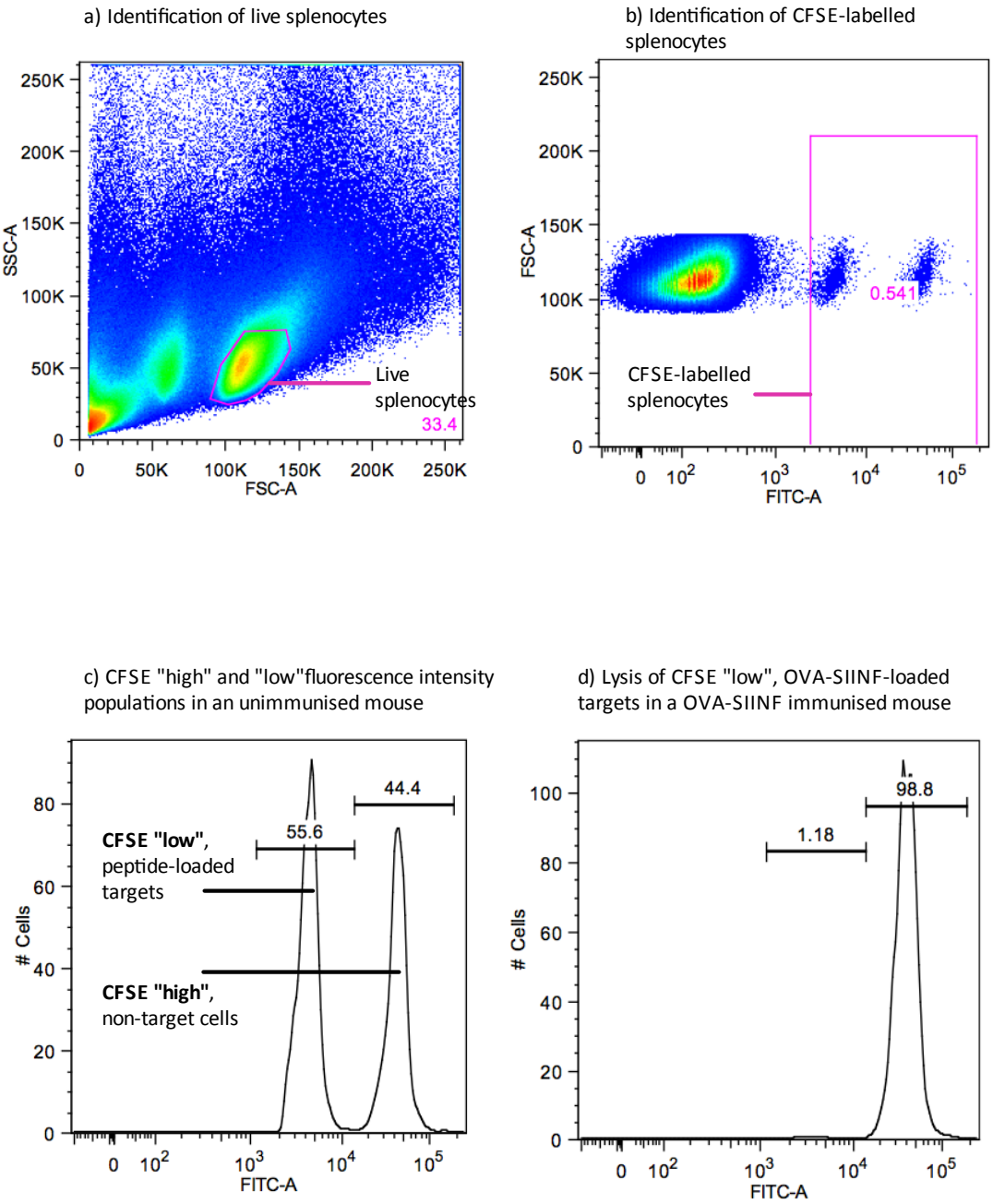
WT1-RMF immunised mouse:

$$\frac{\text{Number of events in the CFSE “low” target population}}{\text{Number of events in the CFSE “high” non-target population}} = \frac{1407}{6043} = 0.23$$

% Target cell lysis = 1- [0.23/1.28] x 100% = **82% lysis of WT1-RMF+ targets**

When possible, at least 2,000 events in the CFSE+ gate were collected, to obtain a reliable reading. Ratios determined in individual unimmunised mice were normalised to the average ratio from 4 unimmunised mice, e.g. 1 - [1.24/1.28] x 100% = 3% lysis of target cells in an unimmunised mouse.

Figure 4-5 Gating strategy for assessment of peptide-loaded target cell lysis in the in vivo cytotoxicity assay.



Eighteen hours post injection of $1x10^7$ CFSE-labelled splenocytes, FACS analysis of CFSE ”low” (target, peptide-loaded) and CFSE “high” (non-target, non-loaded) was performed on splenocytes harvested from immunised and unimmunised mice. Gating of splenocytes and the CFSE labelled populations are shown in (a) and (b). Unimmunised mice served as the control population, demonstrating the presence of both populations of CFSE low and high signal cells, (c). Following 4 rounds of OVA-SIINF immunisation, there was selective destruction of the CFSE low, OVA-SIINF peptide loaded splenocytes, (d).

All immunized mice showed significantly higher lysis of peptide-loaded target splenocytes than unimmunized mice (Figure 4-6). Within the 3 groups of mice immunized against WT1, greatest lysis was seen for the WT1-RMF immunized mice (mean percentage lysis, 74% (56-92%), confidence interval (CI) 95%) compared with mean percentage lysis of 1% (-0.77-2.9%, CI 95%) in unimmunized mice, $p \leq 0.001$. Lysis was seen in each of the WT1-RMF&PADRE (mean lysis 46%, 17-76%, CI 95%) and WT1-YMF&PADRE (mean lysis 49%, 14%-85% CI 95%) immunised groups but at lower mean frequencies than for mice receiving WT1-RMF alone. The lower mean percentage lysis in the groups exposed to PADRE did not reach statistical significance.

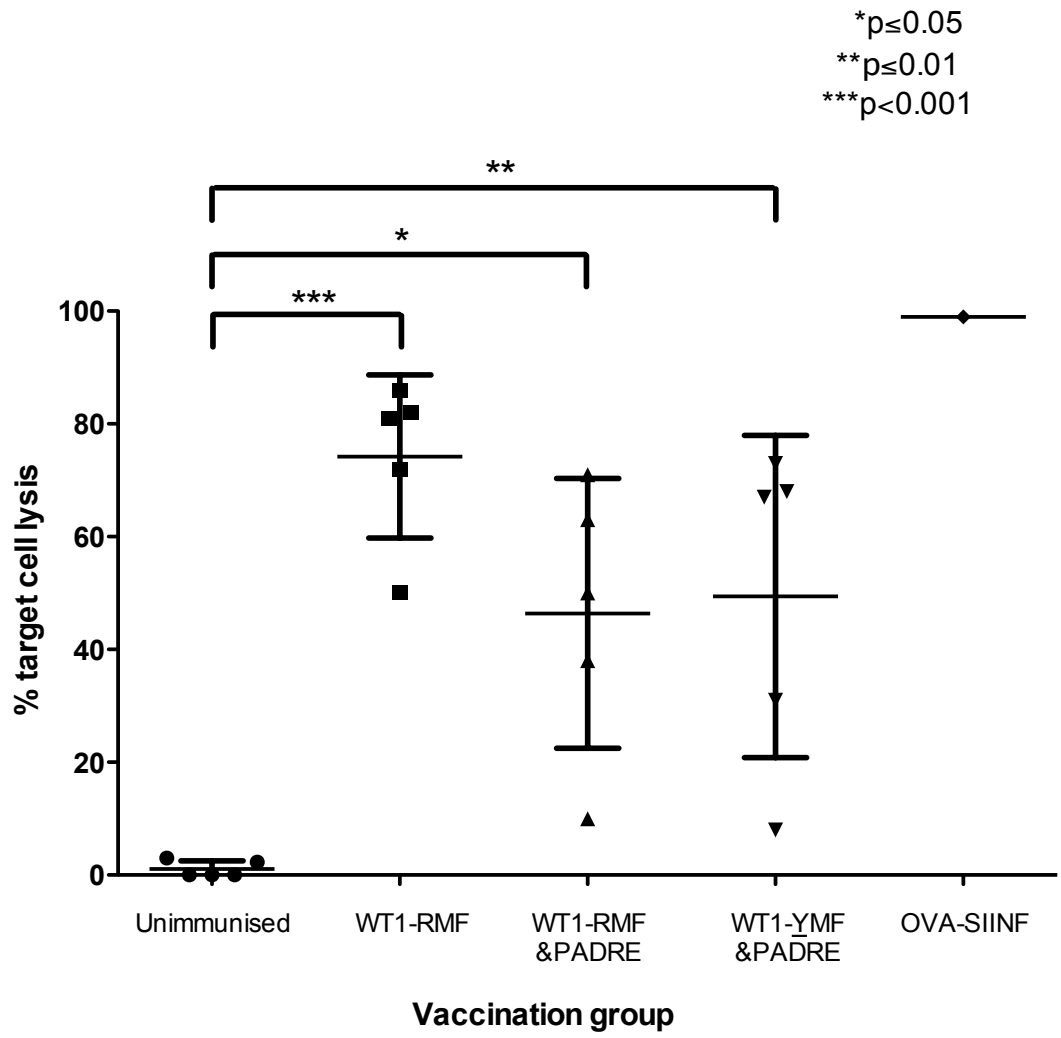
The data from the *in vivo* cytolysis assay contrasts with the findings from pentamer studies in WT1 immunised mice, since only 2/5 mice demonstrated increased frequencies of WT1-RMF pentamer staining T-cells after 3-4 vaccinations, but all 5 mice within that group showed at least 50% lysis of WT1-RMF loaded target cells. Similarly in the WT1-RMF/YMF and PADRE immunised mice, despite the detection of low frequencies of WT1-RMF specific T-cells in 4 of 10 mice by pentamer studies, lysis of WT1-RMF loaded splenocytes was observed in all mice in these 2 groups. One explanation for this apparent discrepancy is that low affinity of the WT1-specific TCR for the WT1-RMF pentamer limits the sensitivity of this assay to detect the expansions of WT1-specific T-cells and therefore underestimates the magnitude of the responses induced. The *in vivo* lysis assay appears to show specificity in that unimmunised mice do not lyse OVA-SIINF or WT1-RMF loaded splenocytes and may be a more sensitive assay for the detection of WT1-RMF specific responses. Furthermore, functionality of WT1-specific cytotoxic T-cell responses may be distinct from the numeric frequency of WT1-specific T-cells.

The possibility that PADRE was impeding the induction of antigen-specific responses was noted. To investigate whether this could have resulted from induction of regulatory CD4+ T-cell expansion triggered through repeated vaccination with a helper epitope, the frequencies of this population in PBMCs isolated from the control and immunised mice were assessed. The gating strategy and results of Treg analyses are shown in Figure 4-7 (a-g). After four rounds of vaccination, no increase in the frequencies of Tregs was detected in mice that received PADRE (Figure 4-7g). In fact, frequencies of Tregs were significantly lower

in PADRE-immunised mice than in all other groups, therefore this was not the explanation for the apparent detrimental effect of PADRE inclusion in vaccinations on immune response induction. To determine if this was a persistent phenomenon, PADRE was evaluated again in a subsequent study, described in 4.2.2.1. Importantly, peripheral blood Treg frequencies were not elevated in any CASAC immunised mice above the observed frequencies in unimmunised mice (Figure 4-7g). This is of relevance, since Treg expansion could suppress the induction of immune responses to a LAA such as WT1, preventing vaccination from having therapeutic efficacy.

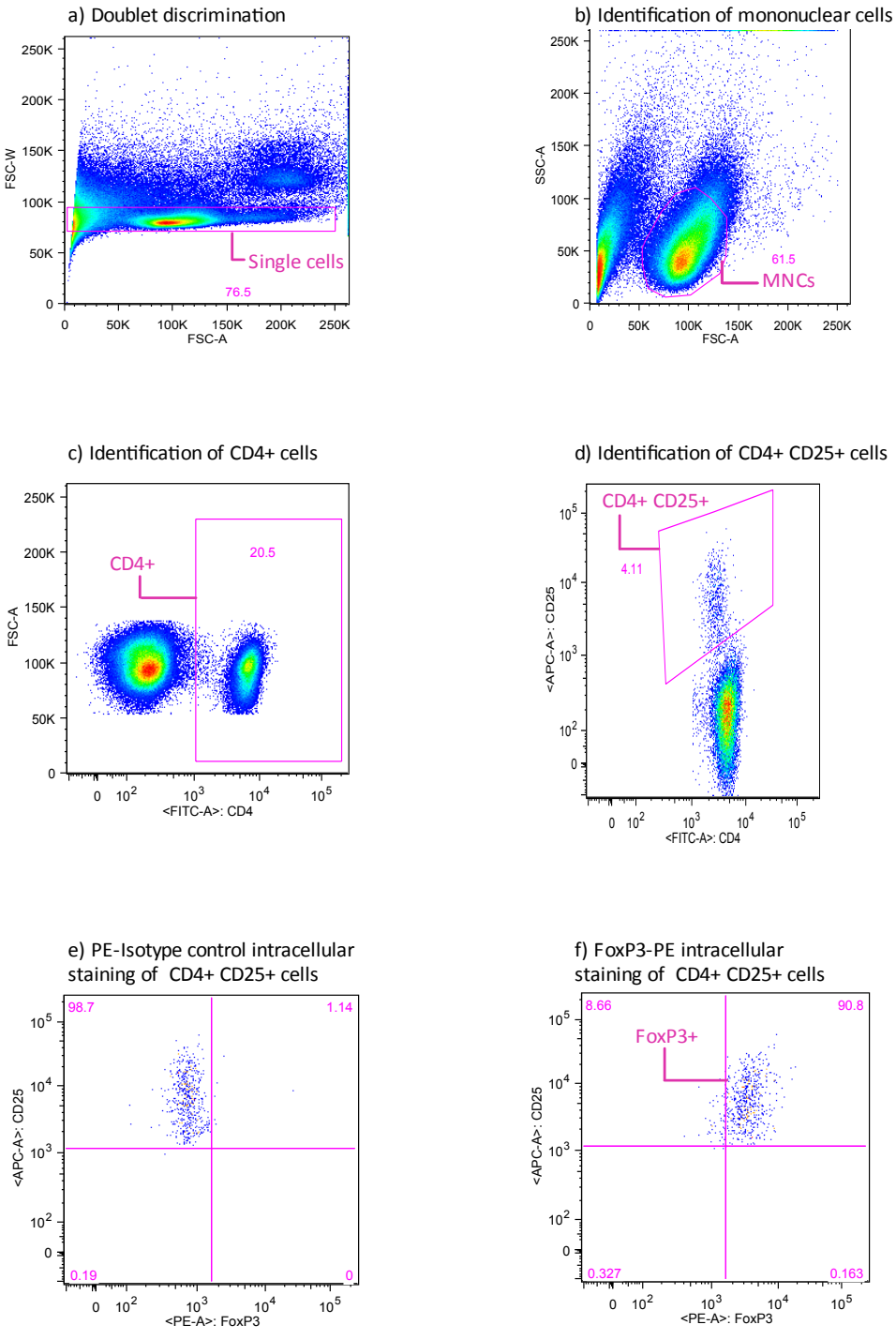
Vaccinations that included WT1-YMF generated antigen-specific T-cell responses that cross-reacted against the native WT1-RMF peptide, as shown in the *in vivo* lysis assay. Such cross-reactivity is vital following immunisation using the heteroclitic peptide, since target tumour cells will present only the native peptide. However, from the results presented so far, the heteroclitic peptide had not been as effective as the native peptide for induction of WT1-RMF-specific immune responses and this became the subject of further investigation (section 4.2.1.2).

Figure 4-6 Potent *in vivo* cytolytic activity following 4 rounds of vaccinations with CASAC and OVA-SIINF or WT1-RMF.



Mean and SD of percentage lysis of peptide-loaded splenocytes following in vivo challenge in each group (n= 5 mice, except for the OVA-SIINF group (positive control) where only 1 mouse was challenged). The assay was performed in the same groups of mice whose antigen-specific T-cell responses were demonstrated in Figures 4-2 to 4-4. Peptides loaded on CFSE "low" target splenocytes were WT1-RMF (for the 3 groups of mice immunised against WT1-RMF/YMF) or OVA-SIINF (for the mouse immunised against OVA-SIINF). One unimmunised mouse was challenged with OVA-SIINF-loaded targets; the remaining 4 were challenged with WT1-RMF-loaded targets. Percentage lysis of target cells by unimmunised mice was used as a negative control and to allow calculation of percentage target cell lysis as outlined in the methods section. A worked example is also provided in the text.

Figure 4-7 No increase in peripheral blood Treg frequencies is observed following repeated CASAC vaccinations



Analysis of Treg frequencies was performed using surface and intracellular fluorescent staining of Treg markers on PBMCs isolated from mice in parallel with monitoring of antigen-specific T-cell frequencies by pentamer studies. Figures 4-6 (a-f) outline the gating strategy. After doublet discrimination (a), the mononuclear population was identified by FSC and SSC properties (b). The CD4+ population was identified (c) and the CD4+ CD25+ sub-population selected (d). A rat IgGκ-PE isotype control antibody was used to determine the gating threshold for expression of FoxP3 (e) prior to analysis of test samples – a representative control from a WT1-RMF & PADRE immunised mouse is shown. Frequencies of CD4+ CD25+ FoxP3+ T-cells as a percentage of CD4+ T-cells were calculated to determine the frequency of Tregs per sample. Figure 4-7g is continued overleaf.

g) Frequency of regulatory T-cells in the peripheral blood of OVA-SIINF or WT1-RMF/YMF & CASAC immunised mice after 4 vaccinations

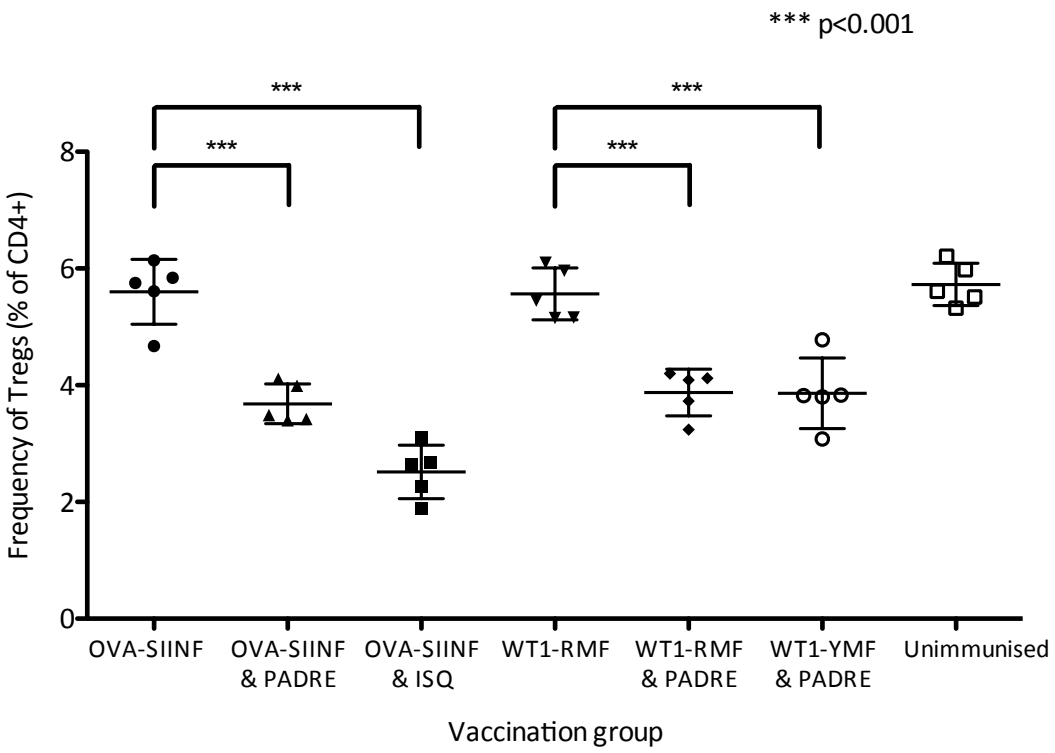


Figure 4-7 continued.

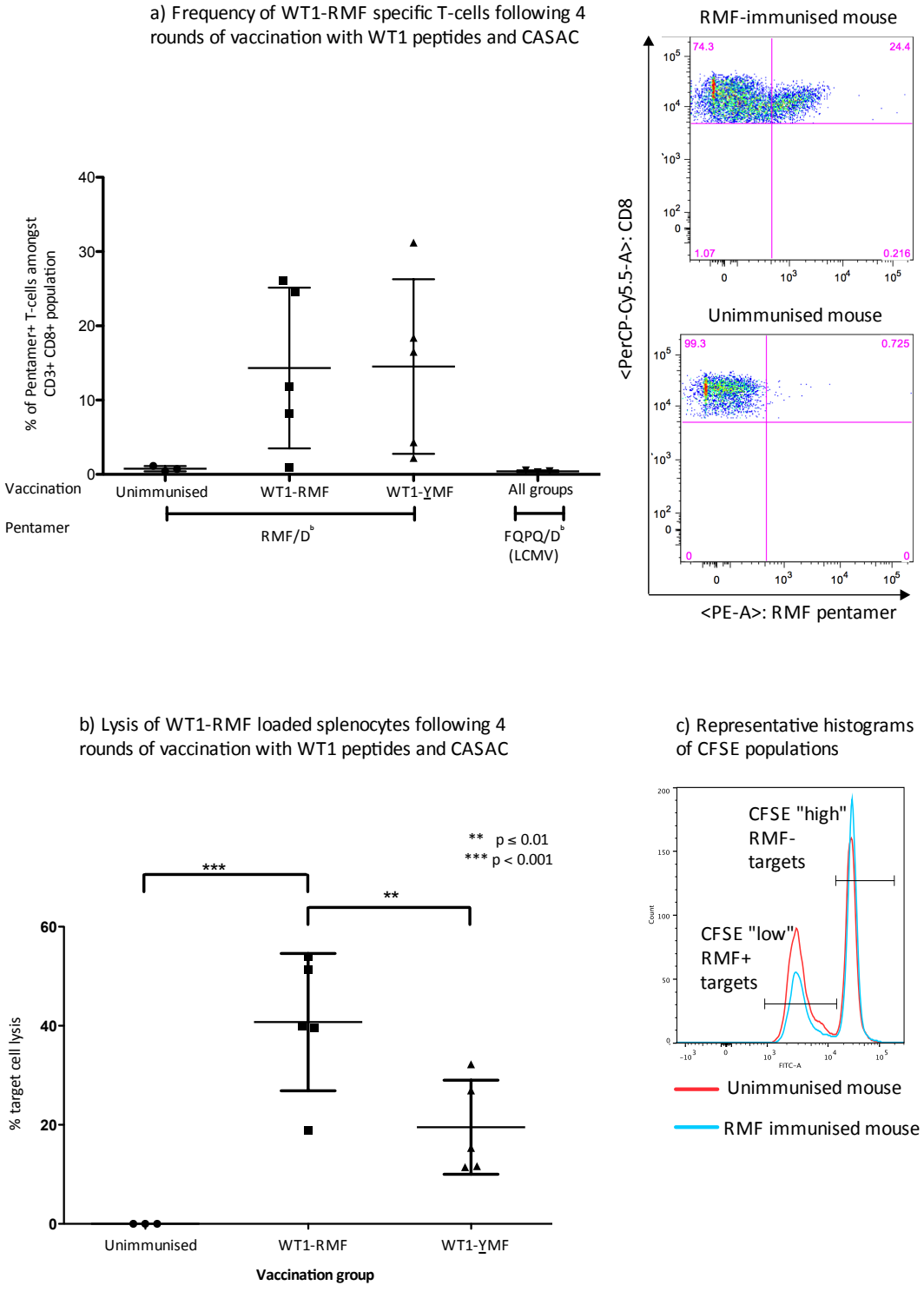
(g) Following 4 rounds of vaccination, Treg frequencies were analysed in each immunisation group; mean and SD are shown for each group in the plot. Lower frequencies of Tregs were observed in the peripheral blood of mice that received PADRE or ISQ in the immunisation in comparison with the corresponding group that did not receive a helper epitope. These differences were statistically significant. Treg, regulatory T-cell; MNC, mononuclear cell; FoxP3, forkhead box P3; PBMCs, peripheral blood mononuclear cells, FSC, forward scatter; SSC, side scatter; IgGk, Immunoglobulin G-kappa

4.2.1.2 Specificity of the immune responses induced following CASAC and WT1 peptide vaccination is demonstrated by peptide specific IFNγ secretion upon stimulation of murine splenocytes

Whilst potent immune responses to peptide vaccinations can arise using CASAC, there is potential for non-specific stimulation of immune cells to occur, leading to off-target activity. To assess this, splenocytes from immunised mice were re-stimulated *in vitro* with either the immunising or an irrelevant peptide and IFNγ production was assessed. Given that the previous study had suggested that addition of PADRE might have limited the magnitude of the immune responses generated with either WT1-RMF or WT1-YMF, a comparison of the effects of using either WT1-YMF or WT1-RMF without PADRE for vaccination was performed in a separate experiment. The specificity of the observed responses was assessed by *in vitro* quantification of peptide-specific IFNγ production by *in vivo* primed T-cells. Groups of mice were immunised four times (days 1, 9, 20 and 27) with CASAC as in the previous experiment (but without any Class II peptides) and either the WT1-RMF or WT1-YMF Class I peptides. *In vivo* cytolysis and intracellular IFNγ assays were performed on days 37 and 38.

Following 4 rounds of vaccination mean frequencies of WT1-RMF specific CD8+ T-cells were 14% in both the WT1-RMF and WT1-YMF immunised groups, demonstrating large expansions of T-cells recognising the native WT1-RMF peptide (Figure 4-8a). However, as seen in the previous study in 4.2.1, some, but not all of the immunised mice from each group showed increased WT1-RMF pentamer staining above background. Despite this, all immunised mice showed lysis of WT-RMF-loaded target cells (Figure 4-8 b, c), although the rate of lysis was variable within groups. As mice were not ear-tagged, it was not possible to link absence of WT1-RMF pentamer staining with reduced lysis of WT1-RMF loaded targets. WT1-RMF-loaded target cell lysis was significantly higher in the WT1-RMF-immunised mice relative to that observed not only in the unimmunised controls but also the WT1-YMF-immunised mice, reflecting the pattern observed in the previous study. Splenocytes harvested at the time of the *in vivo* cytolysis assay were stimulated with relevant or irrelevant peptides *in vitro* and IFNγ production was assessed after 5 hours. The gating strategy for this assay is shown in Figure 4-9 and results of the re-stimulation of WT1-RMF/YMF immunised mice are provided in Figure 4-10. Secretion of IFNγ by splenocytes stimulated *in vitro* was specific to challenge with the immunising/wild type peptide because there was no response to irrelevant peptide.

Figure 4-8 Greater lysis of native WT1-RMF peptide loaded targets by WT1-RMF immunised mice compared with WT1-YMF immunised mice

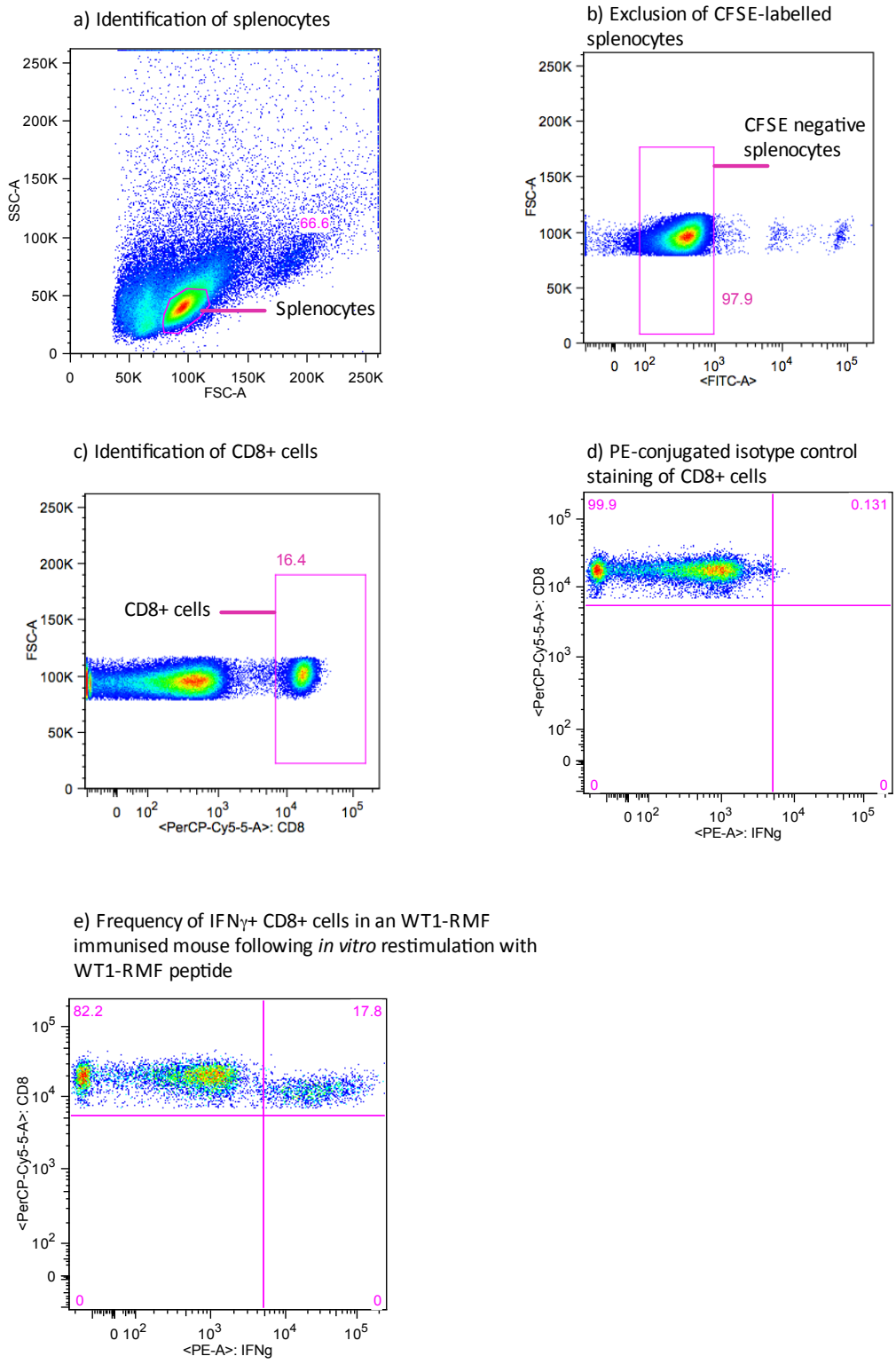


(a) Frequency of WT1-RMF-specific CD8+ T-cells detected following the fourth immunisation for mice immunised with CASAC (no Class II peptide) and either WT1-RMF or WT1-YMF or unimmunised (U) mice (n=3-5 per group). All groups*: staining of a representative sample from each group with irrelevant LCMV-specific FQPQ/D^b pentamer. No significant differences in frequencies of WT1-RMF specific CD8+ T-cells were observed between the immunised and unimmunised groups. Representative WT1-RMF pentamer staining plots are shown in the right panel for a WT1-RMF-immunised mouse (top) and an unimmunised mouse (bottom).
(b) Lysis of WT1-RMF-loaded target splenocytes by the 3 groups following the fourth vaccination.
(c) Representative histograms of CFSE-labelled events obtained in the *in vivo* cytolysis assay in an unimmunised (red) and WT1-RMF immunised mouse.

As demonstrated in Figure 4-10, the proportions of CD8+ cells isolated from the spleens of WT1-RMF and WT1-YMF immunised mice secreting IFNγ following re-stimulation with the native WT1-RMF peptide were significantly higher than background secretion by unimmunised mouse splenocytes. Importantly, cytokine production was not observed when the same cells were challenged with the irrelevant Class I peptide, OVA-SIINF, suggesting specificity of the induced CD8+ T-cell responses.

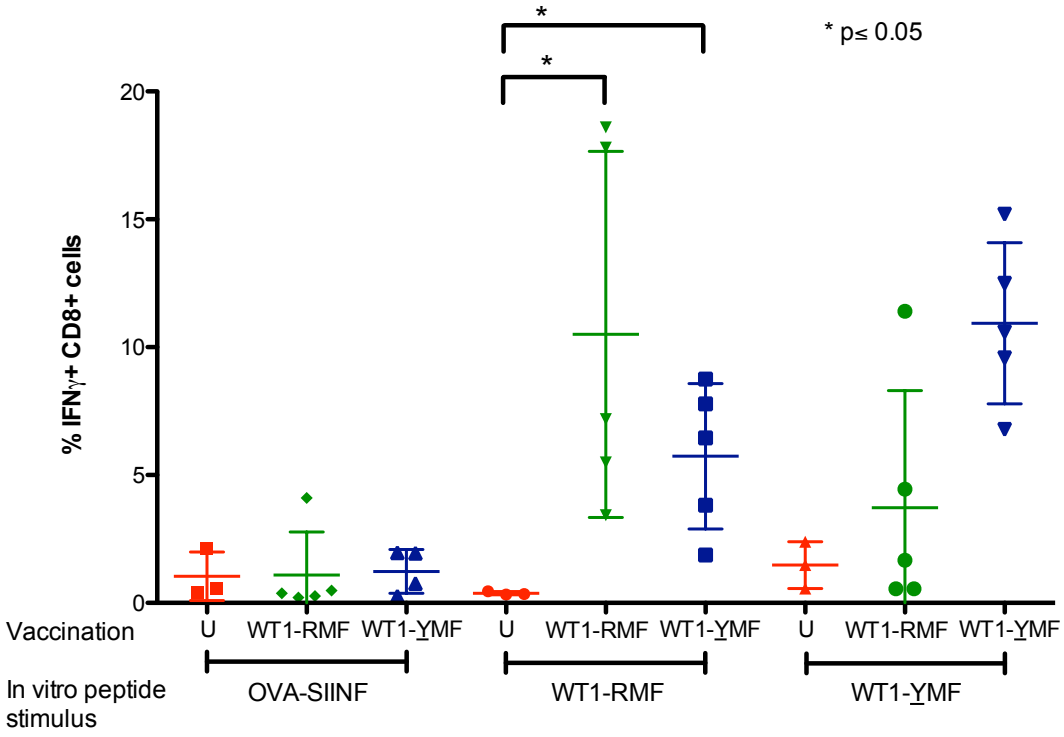
The mean frequencies of IFNγ+ CD8+ T-cells were 10.5% (1.6-19.4%, 95% CI) and 5.7% (2.2-9.3%, 95% CI) upon re-stimulation with WT1-RMF in the WT1-RMF and WT1-YMF immunised groups respectively. Whilst the difference in these means was not statistically significant, the pattern of response replicated the results of the *in vivo* cytolysis assay in Figure 4-8b. Taken together, these observations suggest that the heteroclitic WT1-YMF peptide may not be superior to the WT1-RMF peptide when combined with CASAC to immunise C57BL/6 mice for the induction of cytotoxic T-cell responses against the native WT1-RMF peptide.

Figure 4-9 Gating strategy for intracellular IFNγ assay.



Following the *in vivo* cytolysis assay, splenocytes were harvested and challenged with relevant or irrelevant peptides *in vitro* during a 5-hour intracellular IFNγ assay. (a) Live splenocytes were identified based on FSC and SSC properties. (b) The larger CFSE-negative population is chosen from the live splenocyte population for analysis, to avoid overflow of CFSE-signal into the PE detection channel. (c) CFSE-negative splenocytes were assessed for CD8 expression, by gating on CD8+ cells. (d) PE-conjugated isotype control used to define IFNγ negative population. (e) Example plot demonstrating the presence of a large population (17.8% of CD8+ splenocytes) of IFNγ-producing splenocytes in a WT1-RMF-immunised mouse re-stimulated with WT1-RMF peptide *in vitro*

Figure 4-10 WT1-RMF-specific immune responses can be generated following CASAC and WT1-RMF /YMF vaccination.



Splenocytes were harvested from each group of mice after 4 rounds of vaccination, at the time of the *in vivo* cytotoxicity assay and challenged with either the native WT1-RMF peptide, or the heteroclitic YMF peptide or an irrelevant peptide (OVA-SIINF) in a 5-hour intracellular IFNγ assay. Results for individual mice are shown (along with mean and SD) for each group and peptide stimulus. U, unimmunised control mice.

4.2.1.3 Summary

From these studies, the following conclusions were drawn:

1. Following 2 rounds of vaccination, only a single WT1-RMF and CASAC immunised mouse showed increased WT1-RMF pentamer staining above the background staining in unimmunised mice. No increase in WT1-RMF-specific T-cells was detected in groups that received WT1-RMF plus PADRE or WT1-YMF plus PADRE as the immunising peptides (Figure 4-3). This was in contrast to mice that had been immunised with OVA-SIINF, OVA-SIINF plus OVA-ISQ or OVA-SIINF plus PADRE, although the mean frequencies of OVA-SIINF-specific T-cells in each of the 3 OVA-SIINF-containing groups were not significantly higher than the background percentage detected in the unimmunised mice, due to the within-group wide variation in response magnitudes (Figure 4-2).

2. Following 4 rounds of vaccination, it was possible to detect expansions of WT1-RMF-specific T-cells using pentamer studies in 2 of 5 WT1-RMF-immunised mice (Figure 4-4c,d). This finding suggests that 4 rather than 2 vaccinations are required to induce expansions of WT1-RMF specific T-cells.
3. Within groups of WT1-RMF immunised mice, the rate of response induction was low, with typically 2 responding mice per group. Furthermore, marked variability in the magnitude of immune responses induced (evidenced by pentamer and functional analyses) was repeatedly observed; suggesting that further optimisation of CASAC vaccinations was required.
4. The magnitude of the WT1-RMF specific CD8+ T-cell population detected by pentamer analyses was typically smaller than the OVA-SIINF specific populations observed. This likely reflects the comparative ease of generating immune responses against a xenoantigen in comparison to targeting a self-antigen such as WT1-RMF.
5. Despite detection of low frequencies of WT1-RMF-specific CD8+ T-cells by pentamer analyses after 4 rounds of vaccination in the first experiment, potent lytic capability was observed in all three groups of mice exposed to WT1 peptides and CASAC (Figure 4-6). Underestimation of the size of the WT1-RMF specific T-cell population by pentamer analyses could be due to the lower affinity interaction between the WT1-RMF/D^b complexes and the cognate TCR in comparison with the high affinity interaction existing between OVA-SIINF/K^b and the OVA-SIINF specific TCR, allowing greater ease of detection. Furthermore, even though the frequency of WT1-RMF specific T-cells may be low, the functional potency of this population could be higher.
6. In both OVA-SIINF and WT1-RMF-immunised mice, use of PADRE as the Class II peptide resulted in lower frequencies of antigen-specific responses detected by pentamer studies (significantly lower in the context of OVA-SIINF immunisations). Although differences were not significant, the mean antigen-specific lytic activity also appeared lower for the groups that received PADRE compared with the groups that did not receive this epitope. This response reduction was not due to induction of Tregs, as the use of a helper epitope in vaccinations was associated with lower frequencies of peripheral blood Tregs (Figure 4-7g).
7. The heteroclitic WT1-YMF peptide was not superior in the context of CASAC vaccinations in H-$2D^b$-bearing mice to the native WT1-RMF peptide for induction of WT1-RMF-specific cytolytic activity or IFNγ production following 4 rounds of vaccination.

4.2.2 Strategies aiming to optimise immune response stimulation following CASAC and WT1 peptide vaccination

The data presented in 4.2.1 suggested that it was possible to break tolerance to the self-antigen WT1 by peptide vaccinations targeting WT1, in combination with CASAC. Within groups, a proportion of mice showed antigen-specific responses to WT1 peptide vaccination whilst responses were less pronounced or absent in others, suggesting that the vaccinations were not yet fully optimised. Therefore the next set of studies aimed to enhance the efficacy of immune response induction. The Class I peptide dose, emulsion composition and type of Class II peptide used for immunisations were assessed in subsequent experiments for their effects on WT1-RMF specific immune response stimulation.

4.2.2.1 Vaccinations containing 100μg of WT1-RMF peptide are as effective as vaccinations containing 400μg of WT1-RMF peptide for immune response stimulation

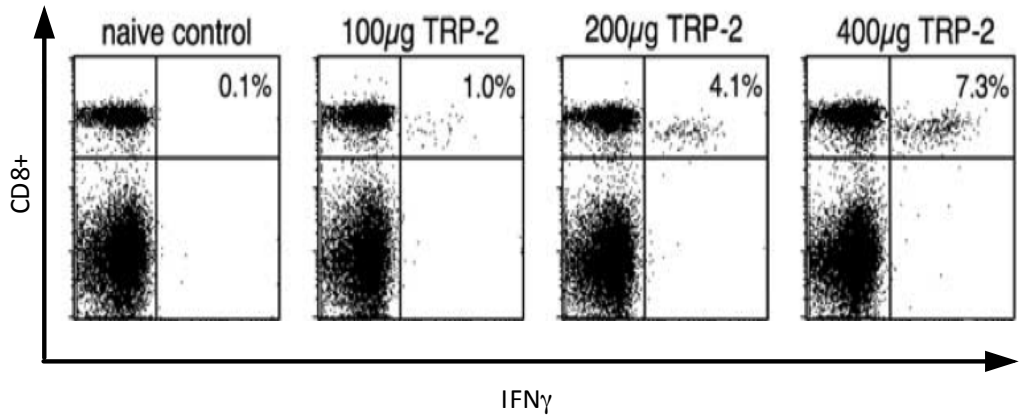
In the original data reported by Wells et al., a peptide derived from the self-antigen tyrosinase-related protein 2, TRP-$2_{180\text{-}188,}$ was analysed in the context of CASAC immunisations. Tolerance to this melanoma-associated antigen could be broken, with induction of TRP-$2_{180\text{-}188}$ specific T-cells following repeated immunisations. Importantly, it was observed that antigen-specific cytokine production in *vitro* by CD8+ cells and *in vivo* cytolytic activity correlated with the use of higher doses (up to 400μg) of TRP-$2_{180\text{-}188}$ in each vaccination, Figure 4-11. Based on these findings, Wells et al. had pursued therapeutic vaccination against B16 melanoma using 400 μg of TRP-$2_{180\text{-}188}$ peptide per mouse[238].

Therefore, it was hypothesised that one means to increase the magnitude and reliability of immune response stimulation following WT1-RMF peptide/CASAC vaccination might be to increase the dose of WT1-RMF peptide per vaccination. This was investigated by immunising groups of mice on days 1, 10, 20 and 33 with either 100, 200 or 400 μg of WT1-RMF peptide. Frequencies of WT1-RMF specific CD8+ T-cells on days 19, 29 and 42 were determined along with *in vivo* cytolytic activity after the fourth round of vaccination (day 45). In parallel, an identical dose escalation study was performed in 3 further groups of mice, but also including the helper PADRE to further investigate the effects of including this helper

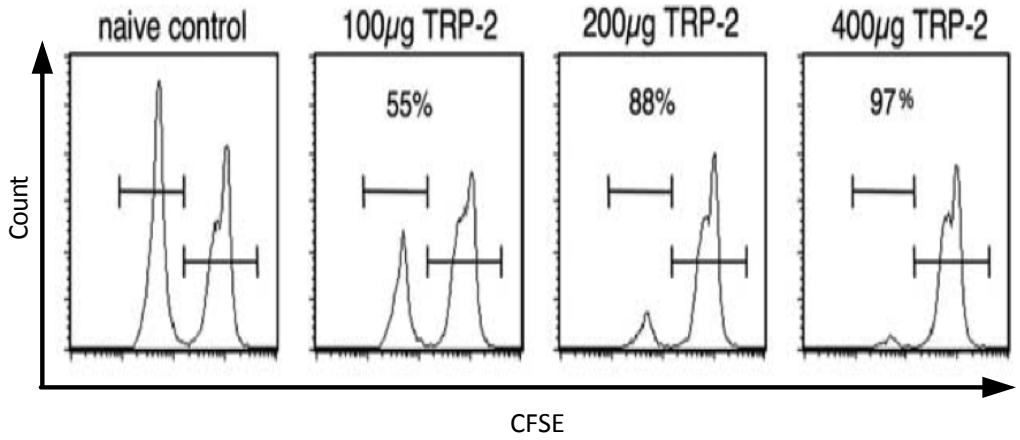
peptide on WT1-RMF specific immune response induction. The results of this dose escalation study are illustrated in Figures 4-12 and 4-13.

Figure 4-11 Greater immune responses to vaccination using TRP-$2_{180-188}$ and CASAC with higher immunising doses of TRP-$2_{180-188}$

a) Increasing immunising dose of TRP-2 Class I peptide correlates with superior IFNγ secretion upon re-stimulation of splenocytes with TRP-2 in vitro



b) Increasing immunising dose of TRP-2 Class I peptide correlates with superior lysis of TRP-2 loaded target cells



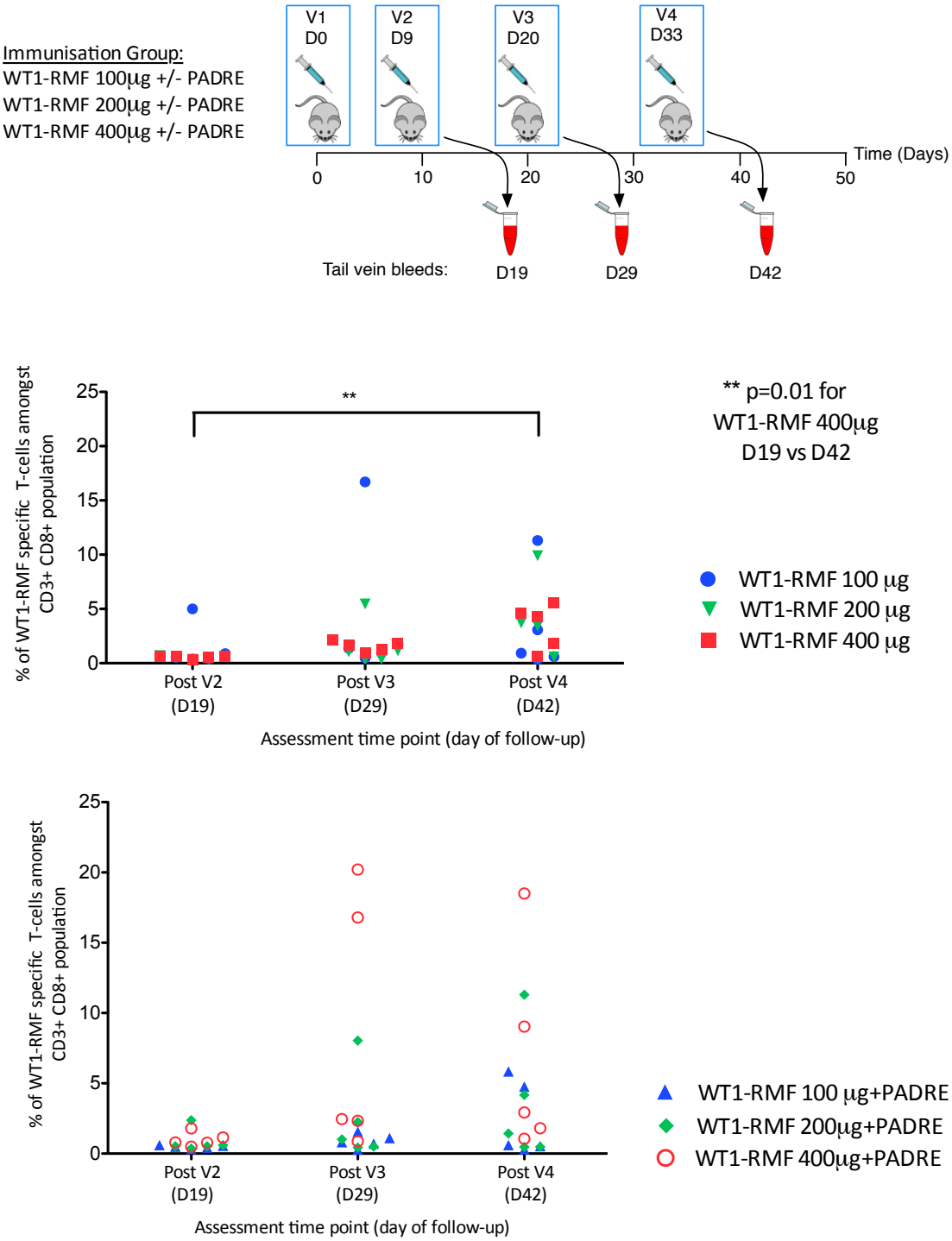
Groups of mice were immunised on days 0, 11 and 19 with either 100, 200 or 400μg of TRP-$2_{180-188}$ and CASAC. (a) IFNγ production by splenocytes isolated from immunised mice after 3 rounds of vaccination and restimulated with TRP-$2_{180-188}$ peptide *in vitro* showed a positive correlation with increasing immunising dose of TRP-$2_{180-188}$.

(b) When *in vivo* CTL activity was assessed, the greatest lysis was discernible at the highest immunising dose of TRP-$2_{180-188}$.

Taken from Wells et al[238].

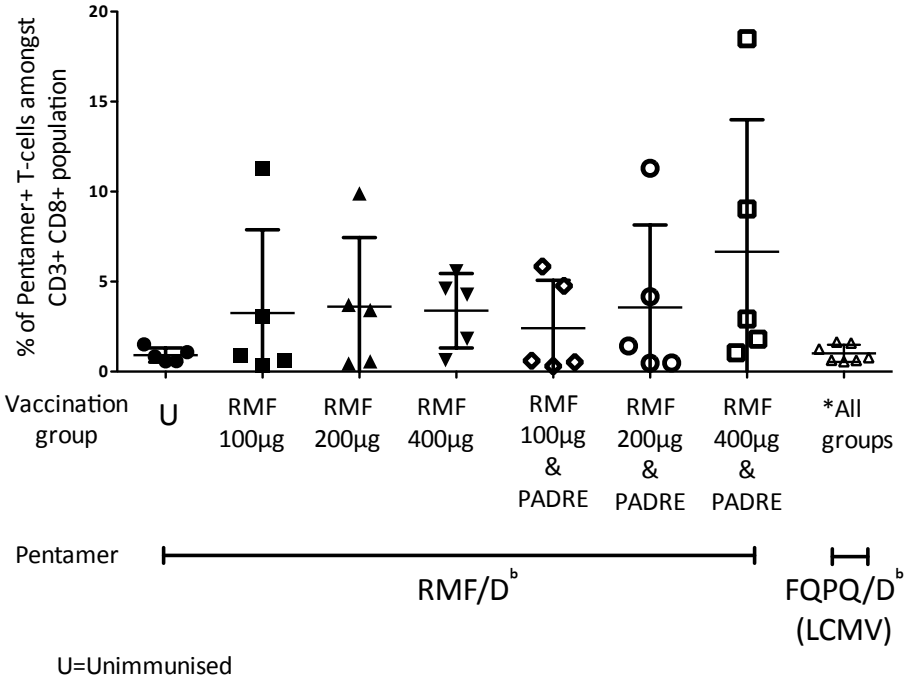
Figure 4-12 Four rounds of CASAC vaccinations using 400μg of WT1-RMF peptide per dose do not induce larger or more potent antigen-specific immune responses in mice than doses containing 100 or 200 μg of WT1-RMF

a) Frequencies of WT1-RMF specific CD8+ T-cells following vaccinations, 2, 3 & 4 in WT1-RMF dose escalation groups



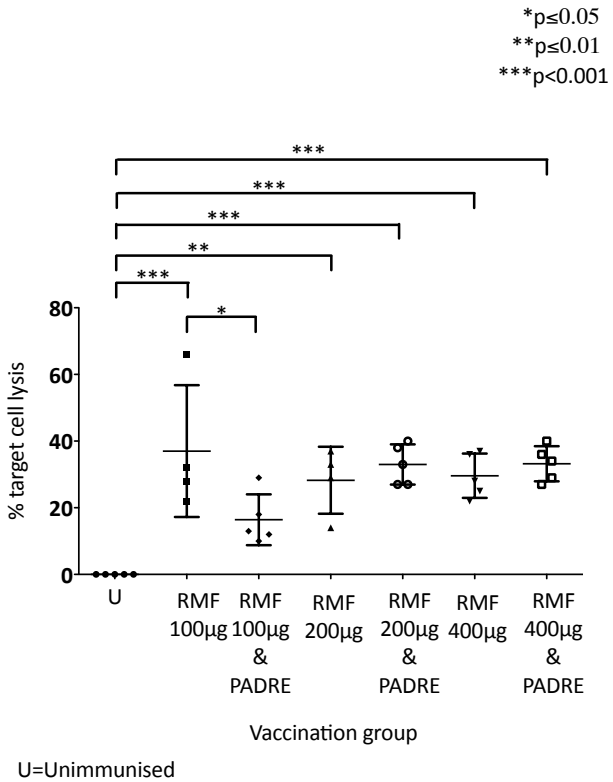
Groups of 5 mice were immunised 4 times (days 0, 9, 20 and 33) with CASAC and either 100μg, 200μg or 400μg of WT1-RMF peptide alone or in combination with 100μg per vaccination of PADRE (schematic). (a) Frequencies of WT1-RMF specific CD8+ T-cells by pentamer studies in WT1-RMF (*upper panel*) and WT1-RMF & PADRE (*lower panel*) immunised mice, after 2, 3 & 4 doses of vaccination. The only significant difference observed was between the mean frequencies of WT1-RMF specific T-cells at days 19 and 42 in mice receiving 400μg of WT1-RMF peptide alone (p=0.01). *All groups: a sample from each group was stained with the with irrelevant LCMV-specific FQPQ/D^b pentamer. V(X), vaccination (number); D, day. Figures (4-12a, this page, 4-12b and c, following page)

b) Frequencies of WT1-RMF specific CD8+ T-cells following 4 rounds of vaccination



U=Unimmunised

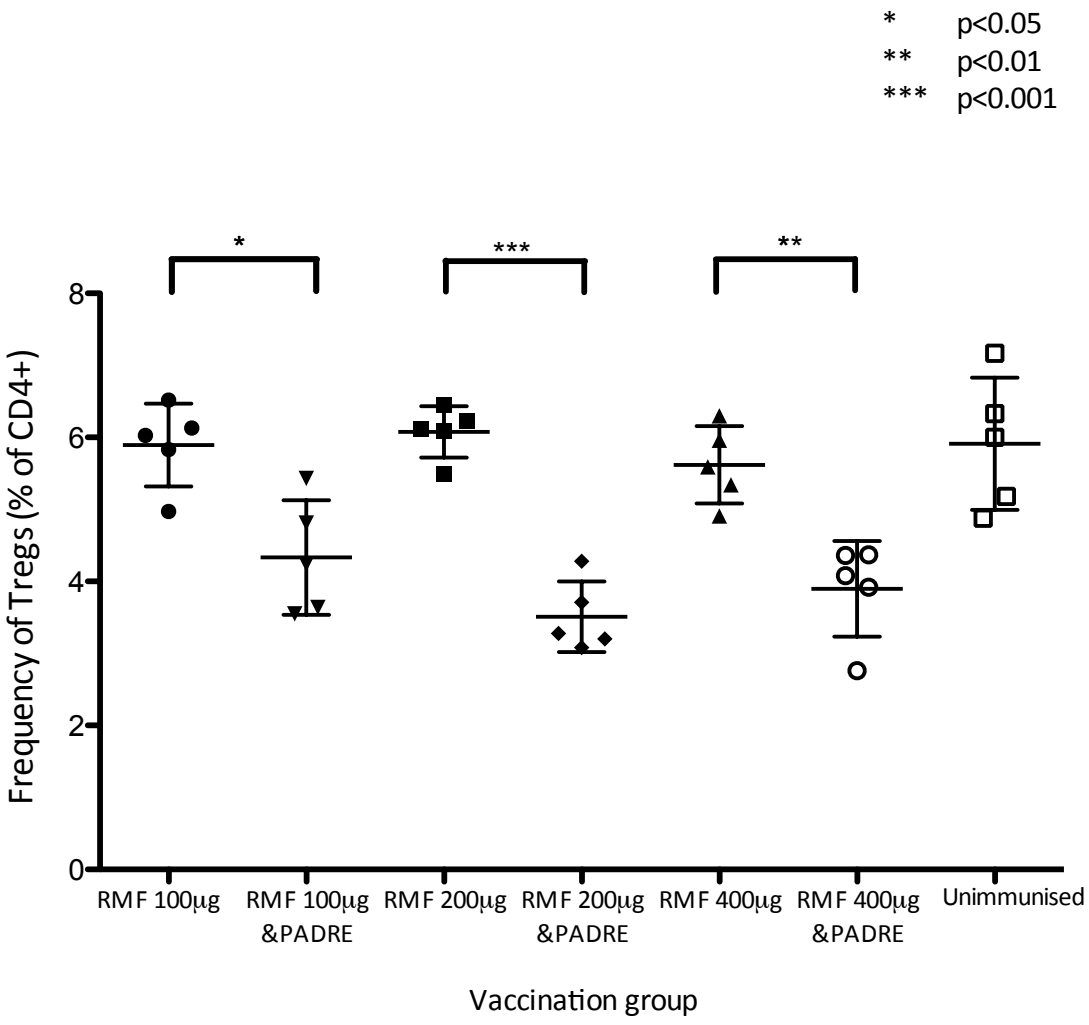
c) Lysis of WT1-RMF loaded target cells after 4 rounds of vaccination



U=Unimmunised

Figure 4-12 continued (b,c)
(b) Individual frequencies, group means and SD of WT1-RMF specific T-cells after 4 rounds of vaccination. No significant differences between groups were detected. (c) Percentage lysis of WT1-RMF loaded target cells following 4 rounds of vaccination in CASAC and WT1-RMF or WT1-RMF & PADRE immunised mice. *All groups: a sample from each group was stained with the with irrelevant LCMV-specific FQPQ/D^b pentamer

Figure 4-13 Reduction in frequencies of peripheral blood regulatory T-cells following 4 vaccinations combining WT1-RMF and PADRE



Frequencies of Tregs following 4 rounds of vaccination were determined in parallel with the pentamer analyses in the same mice described in Figure 4-12. Frequencies of peripheral blood Tregs along with mean and SD are depicted.

In contrast to the studies of TRP-$2_{180-188}$ vaccinations[238], no significant differences with respect to frequencies of WT1-RMF specific CD8+ T-cells were observed using higher doses of WT1-RMF peptide for immunisation (Figure 4-12). A significant increase in lysis of WT1-RMF loaded target cells above the background observed in unimmunised mice was observed in all groups except the mice receiving WT1-RMF 100μg and PADRE, a pattern of response which was similar to the findings comparing WT1-RMF (100μg) with WT1-RMF (100μg) and PADRE, demonstrated in Figure 4-6. There was no significantly higher percentage lysis of WT1-RMF-loaded targets in the assay in mice vaccinated with 400 μg of WT1-RMF, despite the suggestion of more uniform response induction within this group.

While no statistically significant differences in the magnitude of the immune responses observed between the three groups of mice receiving WT1-RMF and PADRE were observed, the higher mean frequency of WT1-RMF specific T-cells in the group of mice receiving 200-400μg of WT1-RMF suggests that this higher dose may have been able to overcome any negative impact of the inclusion of PADRE (Figure 4-12b). This may be due to competition for uptake by APCs between WT1-RMF and PADRE. A "family" of PADRE epitopes was initially designed to bind universally to MHC Class II molecules ($PADRE_{760}$, $PADRE_{906}$ and $PADRE_{965}$ peptides). $PADRE_{965}$ was reported to have good binding affinity for IA^b, the C57BL/6 Class II molecule and therefore was used in the vaccination studies presented here. $PADRE_{760}$ and $PADRE_{906}$ were described as being unable to bind to human HLA Class I molecules in the original publication by Alexander et al, although binding affinity of $PADRE_{965}$ to HLA-Class I/H-$2D^b$ was not discussed[266]. Therefore competitive binding of Class I epitopes processed from $PADRE_{965}$ to H-$2D^b$ may provide an explanation for the results presented herein. Analysis of regulatory T-cell frequencies in mice receiving vaccinations containing PADRE again demonstrated a significant reduction in peripheral blood Tregs relative to the corresponding groups not exposed to PADRE (Figure 4-13), similarly to the earlier findings described in 4.2.1, Figure 4-7. Together these observations suggest that Treg-mediated suppression is not responsible for the lower magnitude of immune response induction following PADRE and CASAC vaccinations.

Both this study and that reported in 4.2.1 (Figure 4-4) demonstrated no significant differences in the mean frequencies of antigen-specific T-cells after 3 or 4 rounds of vaccination. However, in one condition

in this set of vaccinations (CASAC vaccination using WT1-RMF 400μg alone, Figure 4-12a) there was a significant difference between frequencies of WT1-RMF specific T-cells between the day 42 assessment (mean 3.38% WT1-RMF specific T-cells, 0.82-5.95%, CI 95%) and the day 19 assessment (mean 0.53% WT1-RMF specific T-cells, 0.36-0.7%, CI 95%, p=0.01). Given that frequencies of antigen-specific T-cells did not increase after dose 3 or 4, it was decided to analyse antigen specific T-cell frequencies in subsequent experiments after the second and third vaccinations with functional assessment alone performed after the fourth vaccination. This reduced the number of procedures performed in the mice.

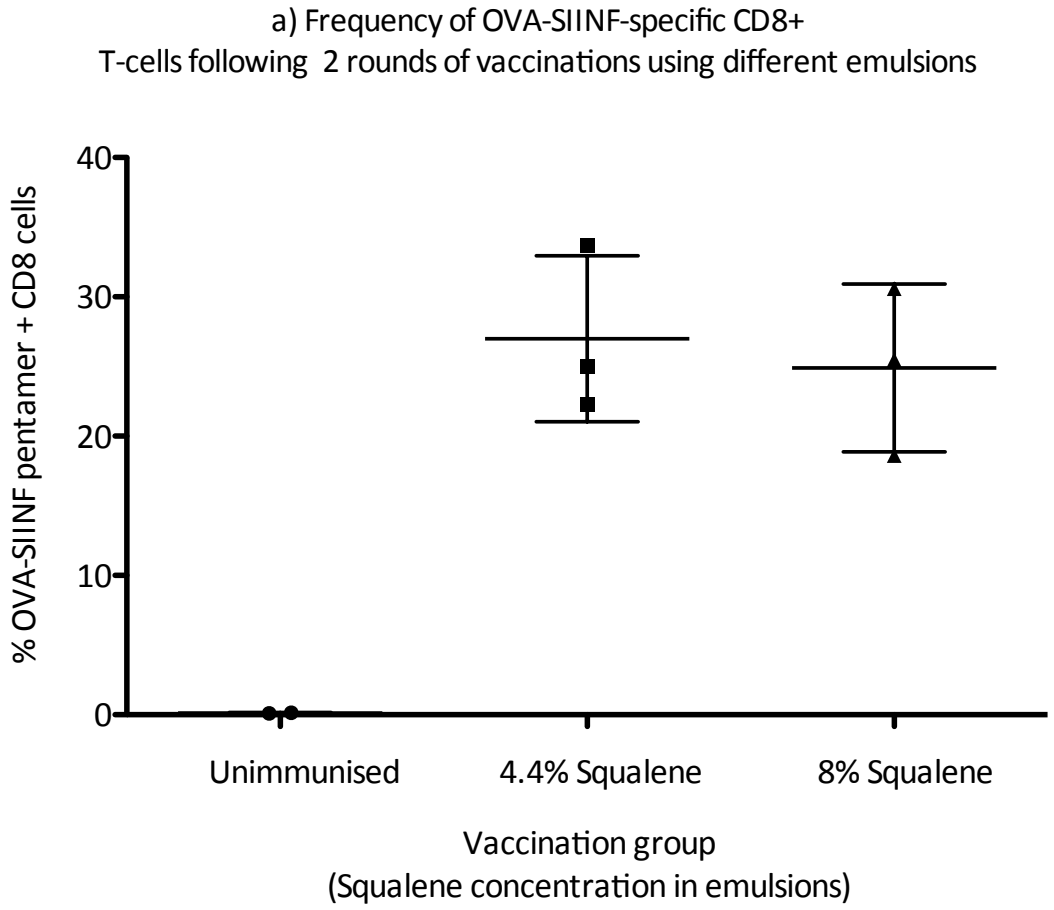
4.2.2.2 Emulsion composition may affect efficacy of CASAC/WT1-RMF vaccinations: effects of varying squalene concentration

CASAC vaccinations targeting OVA-SIINF result in induction of potent immune responses with a high frequency of responding mice. Conversely, whilst antigen-specific immune responses can be generated following CASAC and WT1-RMF vaccinations, variability of the induced responses suggests that fine-tuning of vaccine components might improve immune response generation. Wells et al. suggested that the presence and quantity of squalene in the emulsion did not impact upon the efficacy of CASAC vaccinations targeting OVA-SIINF; only the requirement of 2 TLR agonists and Tween 80 for effective immune response generation was essential[238]. Squalene is included in the emulsion as the oily phase of this oil-in-water (o/w) emulsion[238,285]. The CASAC emulsion itself is similar in composition to MF59®, which forms a stable emulsion in clinical grade vaccine, increases bioavailability of vaccine components and appears to promote induction of an immunocompetent milieu at the vaccination site[285]. It is possible therefore, that squalene composition of the emulsion could still be relevant for immunisations targeting less immunogenic epitopes than OVA-SIINF. Therefore, whether squalene composition might affect vaccination efficacy targeting WT1-RMF was evaluated by testing 3 different squalene concentrations in separate emulsions: 2.2% v/v, 4.4% v/v (used in the Wells paper) and 8% v/v.

Consistent with the data reported by Wells et al., there was no effect of altering the squalene composition of the emulsions on generation of OVA-SIINF-specific CD8+ T-cell responses (Figure 4-14a). *In vivo* cytolysis was not assessed for these OVA-SIINF immunised mice as experience using this vaccination

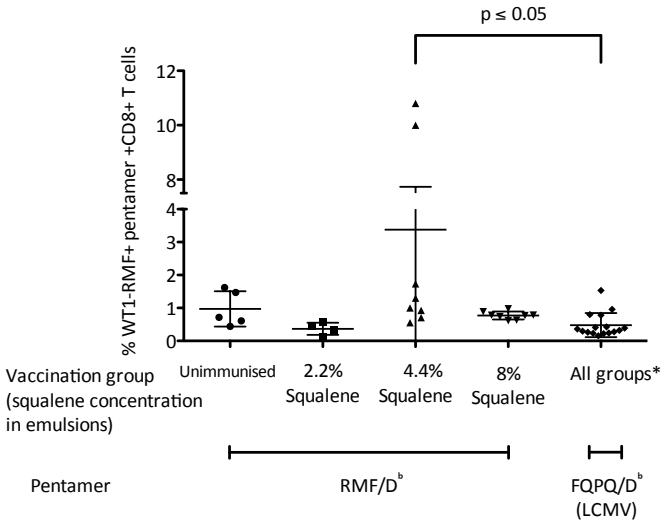
suggested that with the observed percentages of OVA-SIINF-specific T-cells, lysis of OVA-SIINF-loaded splenocytes would be vigorous.

Figure 4-14 Reduced magnitude of WT1-RMF specific, but not OVA-SIINF specific, immune response induction when combined with CASAC containing 8% squalene v/v emulsion



Groups of mice were immunised with OVA-SIINF and CASAC (Figure 4-14a, this page) or WT1-RMF and CASAC (Figure 4-14b,c, following page) vaccines containing 2.2%, 4.4% or 8% v/v emulsions. (a) Following 2 vaccinations, no significant difference in the frequencies of OVA-SIINF specific CD8+ T-cells were observed following OVA-SIINF vaccinations using either 4.4% or 8% squalene v/v emulsions. Mean and SD are shown for each group.

b) Frequency of WT1-RMF-specific
T-cells following the 3rd vaccination, using different emulsions



c) Lysis of WT1-RMF loaded targets following the 4th vaccination, using different emulsions

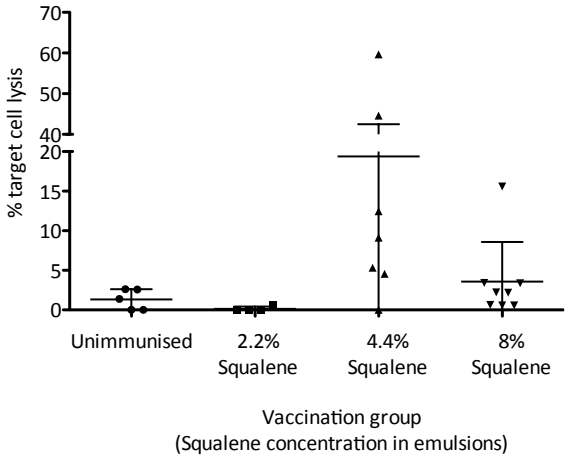


Figure 4-14 continued (b,c)

Results of WT1-RMF pentamer analyses following 3 rounds of vaccination are combined from 2 similar experiments with *in vivo* lysis assay performed in a subset of these mice. (b) Frequencies of WT1-RMF specific CD8+ T-cells following the third vaccination targeting WT1-RMF using CASAC with each emulsion type are shown. *All groups: staining of representative samples from each group with irrelevant control LCMV pentamer. (c) Percentage lysis of target WT1-RMF-loaded splenocytes following the fourth vaccination in a subset of mice (2.2% squalene, n=4, 4.4% squalene, n=7, 8% squalene, n=8). Mean and SD are shown for each group; no statistically significant differences in lysis of WT1-RMF loaded target cells between the immunised groups were observed.

However, in WT1-RMF/CASAC immunised mice, using 2.2%, 4.4% and 8% emulsions in 2 separate but similar experiments (Figure 4-14b,c) altering the squalene composition affected the magnitude of the immune response generated (as demonstrated by pentamer studies and *in vivo* cytolysis).

A significantly higher mean frequency of WT1-RMF specific CD8+ T-cells was observed in the 4.4% v/v squalene emulsion group above the background staining observed with the irrelevant control pentamer, although statistically significant differences between groups receiving emulsions with varying squalene concentration were not detected (Figure 4-14b). These findings suggest that composition of the vaccine emulsion may differ according to the target peptide, highlighting the importance of detailed evaluations of individual peptides used within the context of CASAC.

4.2.2.3 A WT1-derived Class II peptide, an unrelated Class II peptide or agonist anti-CD40 antibody may combine with WT1-RMF/CASAC to induce WT1-specific CD8+ T cell responses

The data presented by Wells et al stated that inclusion of either a Class II peptide, related or unrelated to the target Class I peptide, or an agonist anti-CD40 antibody within CASAC could effectively promote OVA-SIINF specific CD8+ T-cell expansion following repeated vaccination[238]. However, studies presented earlier, using PADRE as a helper peptide, had not demonstrated superior induction of immune responses in groups receiving this peptide in the context of CASAC vaccinations. The OVA-derived Class II helper peptide OVA-ISQ was shown to combine with OVA-SIINF to enhance expansion of OVA-SIINF specific CD8+ T-cells in the data presented by Wells et al, although a direct comparison of vaccinations targeting OVA-SIINF using CpG, poly I:C, IFNγ with or without OVA-ISQ was not described[238]. Inclusion of an effective Class II helper peptide in vaccinations could aid the generation of a long-term memory response[210,211,286], as demonstrated by the strong recall responses observed in mice previously immunised with OVA-SIINF and CASAC more than 100 days earlier[238].

Whilst OVA-ISQ may be an effective Class II helper peptide in the context of the experimental mouse model, more clinically relevant Class II peptides for use in CASAC vaccinations targeting WT1 in humans are ultimately desirable. A long Class II helper peptide PGCNKRYFKLSHLQMHSRKHTG (WT1-PGC) used in a

clinical trial of WT1 peptide vaccination[201], homologous in humans and mice and predicted to bind to I-A^b using the immune epitope database (IEDB)[181] (see Table 2-3), was therefore evaluated in CASAC vaccinations targeting WT1-RMF. OVA-ISQ was used as a comparator for provision of Class II help due to its demonstrable compatibility with CASAC. Vaccinations using WT1-RMF without a class II peptide, shown earlier to induce detectable WT1-RMF-specific T-cells and promote significant lytic activity, were also included as positive controls.

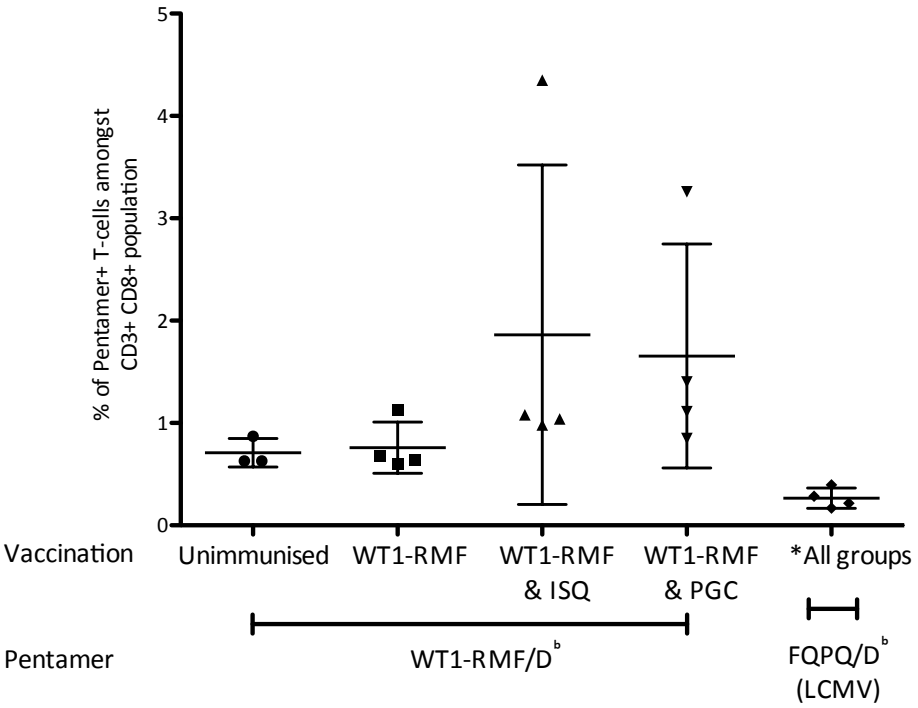
Vaccinations were delivered on days 1, 11, 21 and 29 with analysis of WT1-RMF CD8+ T-cell frequencies on days 15, 26 and *in vivo* cytolytic analysis on day 35. The results are shown in Figure 4-15. No statistically significant enhancement of WT1-RMF specific CD8+ T-cell expansion or lysis of WT1-RMF loaded targets was observed with the inclusion of either OVA-ISQ or WT1-PGC in vaccinations (Figure 4-15a-b). However, as seen previously, 1-2 mice within each of the groups receiving a helper epitope exhibited robust responses but there was considerable variability in immune response stimulation against WT1-RMF using CASAC. Although this particular study suggests there may be an advantage to include helper epitopes in vaccinations, this is only supported by small numbers of responding mice in these groups and uniformity of responses was not demonstrable. *In vitro* re-stimulation was performed using either the same Class I or II peptides used for immunisations or peptides which had not been included in the vaccines and to which CD4+ and CD8+ T-cells would be naïve (negative controls). These results demonstrated the specificity of the responses generated, with higher frequencies of IFNγ-producing CD8+ and CD4+ T-cells respectively upon re-stimulation with the same peptides used at immunisation (Figure 4-15 c-d). Of note, the *in vitro* CD4+ IFNγ responses following re-stimulation with OVA-ISQ are robust whilst responses to WT1-PGC are much lower (Figure 4-15d). This suggests that WT1-PGC has low helper activity in the context of WT1-RMF and CASAC vaccinations in C57BL/6 mice.

Overall, lower WT1-RMF CD8+ T-cell expansion and functional activity were observed in these experiments compared with earlier studies presented in 4.2.1. No change in suppliers of peptides or CASAC constituents, or methods of reconstitution or storage, took place to account for the diminished responses. Freeze-thaw cycles were also kept to the absolute minimum in order not to compromise

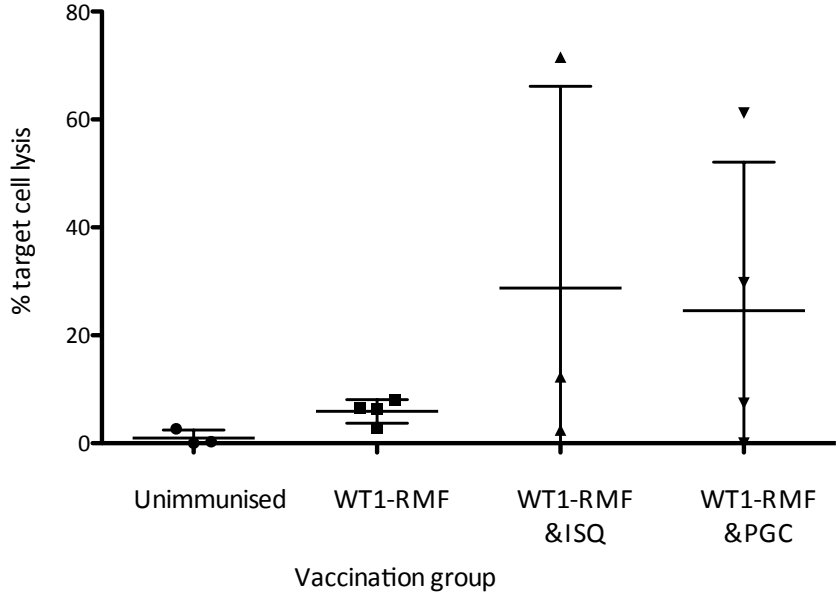
peptide stability. The WT1-RMF peptide was able to bind stably to HLA-A2-expressing T2 cells, suggesting that it was intact (even after freezing and thawing, Figure 4-16). To ensure that CASAC components were functional, in all subsequent experiments, OVA-SIINF and CASAC immunised mice were included as positive controls since an expected and predictable response with CASAC immunisations using this peptide should be observed.

Figure 4-15 (a-d) A relevant or irrelevant helper peptide may be used to promote WT1-specific immune responses following CASAC vaccination

a) Frequency of WT1-RMF specific CD8+ T-cells following 3 rounds of vaccination

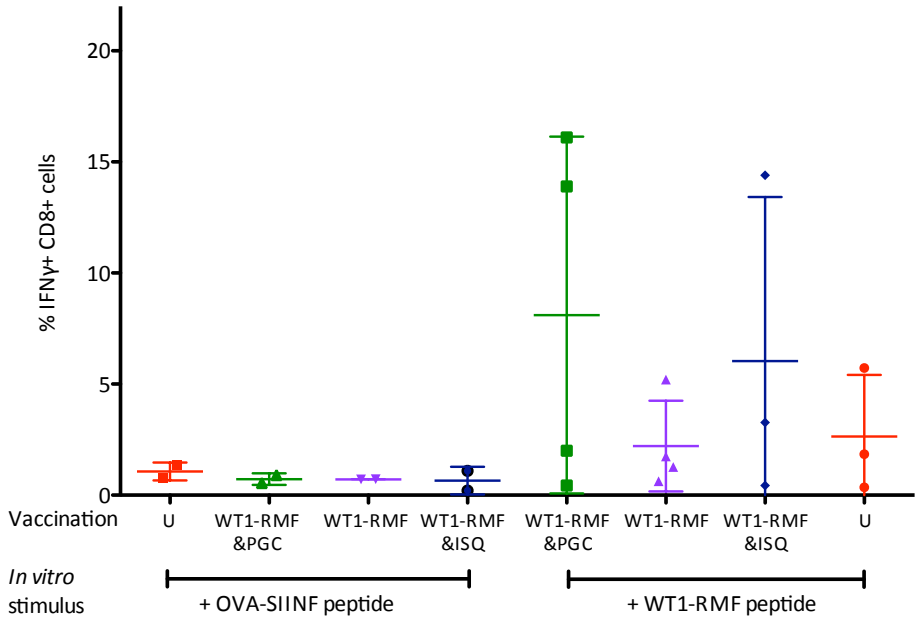


b) *In vivo* lysis of WT1-RMF loaded traget-cells following 4 rounds of vaccination



Groups of up to 4 mice were immunised 4 times with WT1-RMF and CASAC alone or in combination with WT1-PGC or OVA-ISQ. (a) Results of pentamer analyses following the third vaccination are shown. *All groups: a representative sample, from each group, was stained with the irrelevant LCMV pentamer. (b) After the fourth vaccination, in vivo lysis of WT1-RMF-loaded splenocytes was assessed. One mouse in the WT1-RMF&ISQ group died during the *in vivo* CTL assay and therefore was lost to analysis. Mean +/- SD for each group is shown. No statistically significant differences were observed between any of these groups in either experiment. Figure 4-15 continues overleaf.

c) Frequency of IFNγ + CD8+ T-cells in WT1/CASAC immunised mice following 4 rounds of vaccination after *in vitro* restimulation



d) Frequency of IFNγ + CD4+ T-cells in WT1/CASAC immunised mice following 4 rounds of vaccination after *in vitro* re-stimulation

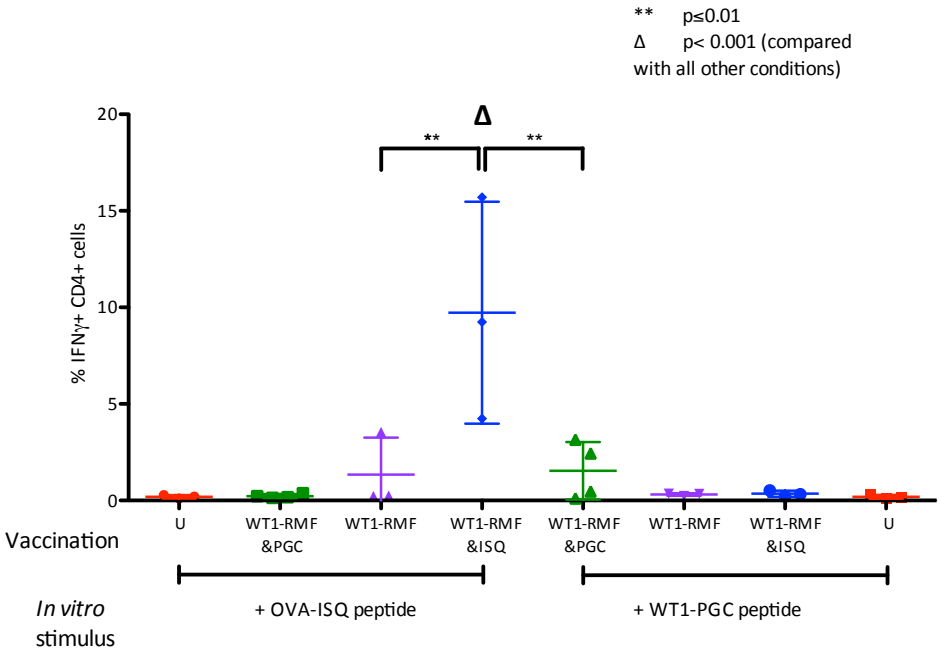
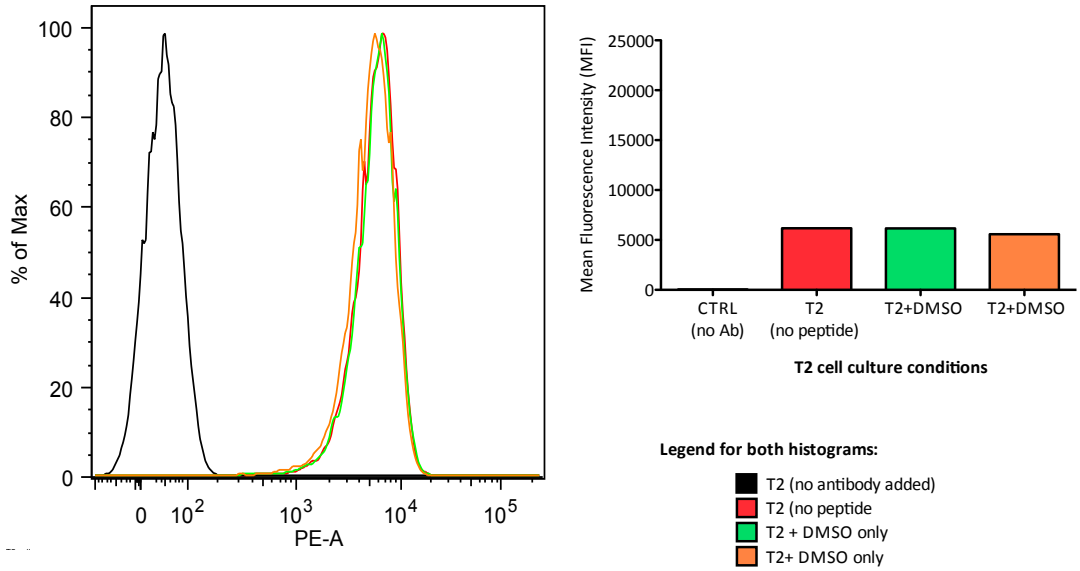


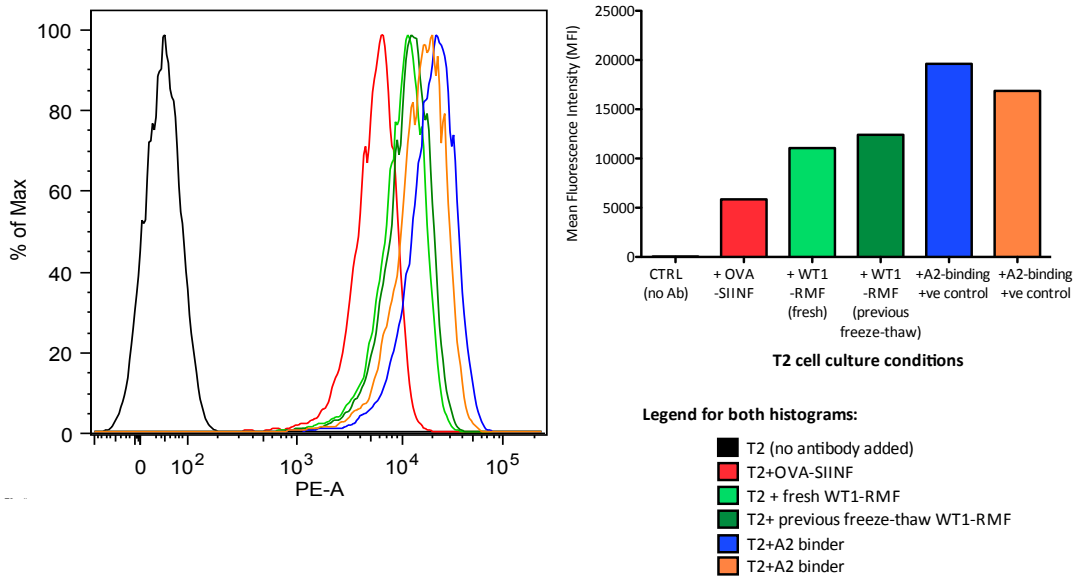
Figure 4-15 continued.
Splenocytes harvested from the same mice described in Figures 4-15 a and b were re-stimulated in a 5 hour intracellular IFNγ assay with WT1-RMF (or OVA-SIINF as an irrelevant peptide) to assess IFNγ production by CD8+ T-cells (c) or with OVA-ISQ or WT1-PGC to assess IFNγ production by CD4+ T-cells (d). Mean +/- SD for each group is shown. No significant differences between groups were observed with regards to IFNγ production by CD8+ T-cells.

Figure 4-16 Binding of WT1-RMF peptide even following previous freeze-thaw cycles allows stabilisation of HLA-A2 expression by TAP-defective T2 cells

a) Low surface expression of HLA-A*02:01 by T2 cells in the absence of exogenous HLA-A*02:01-binding peptides



b) Surface expression of HLA-A*02:01 by T2 cells is increased by addition of HLA-A*02:01-binding peptides, including the WT1 peptide used for vaccinations.

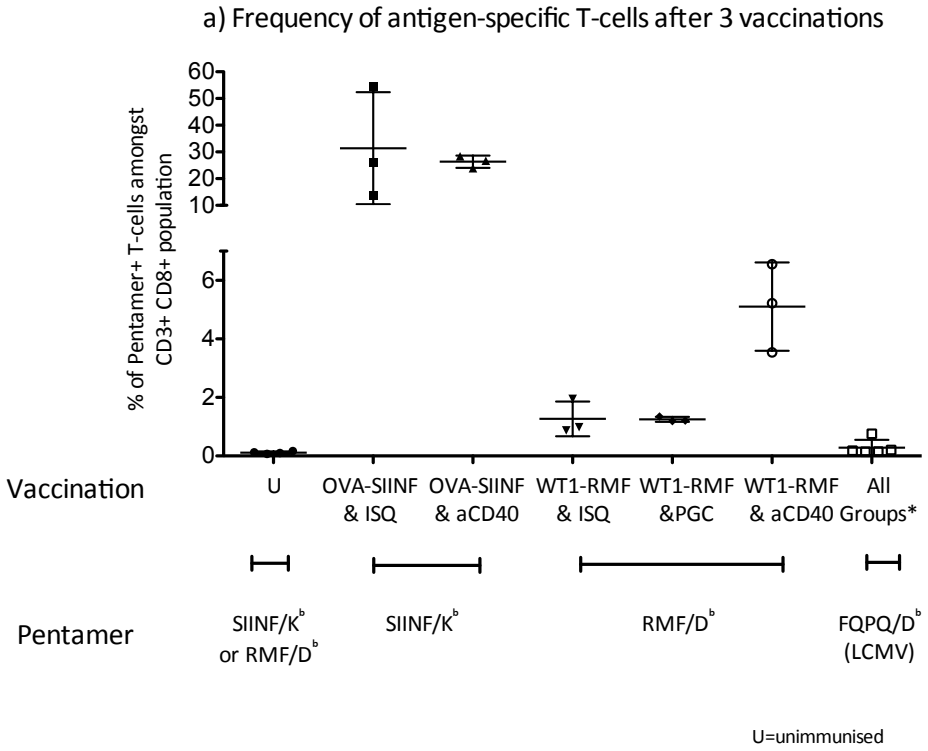


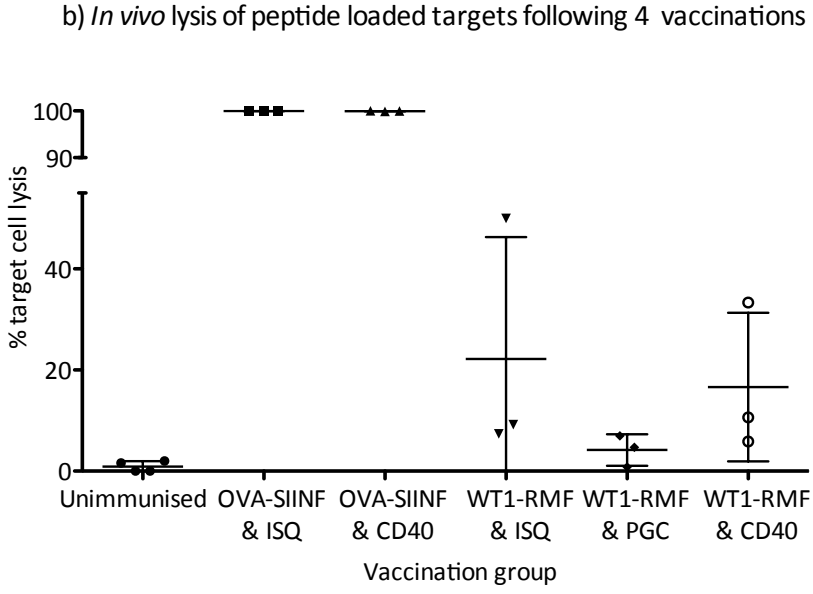
The ability of either freshly prepared or previously frozen and thawed WT1-RMF peptide to stabilise HLA-A2 expression by T2 cells was used as a surrogate of persisting peptide stability. $5x10^5$ T2 cells were cultured overnight in the presence of known HLA-A2 binding peptides (YLL, NLV or WT1-RMF) or the non-A2 binding OVA-SIINFEKL peptide, or no peptide. The next day, cells were single-stained with anti-HLA-A2-PE and the MFI of the bound antibody determined by FACS. (a) Histogram demonstrating the relative HLA-A2 expression profiles for the different peptides or T2 cells in the absence of antibody; the MFI is also depicted per cell type in the accompanying bar chart. (b) Histogram demonstrating the relative expression of HLA-A2 by T2 cells cultured in the absence of peptide or presence of DMSO alone as negative controls. T2, Transporter for Antigen Processing (TAP)-defective cells; MFI, median fluorescence intensity; YLL, **YLL**PAIVHI, RNA-dependent helicase$_{148–156}$ peptide; NLV, **NLV**PMVATVQ derived from cytomegalovirus structural protein pp65, DMSO, dimethyl sulfoxide

In the absence of a Class II helper peptide, direct ligation of CD40 on DCs may be achieved by using an agonist anti-CD40 antibody (rather than relying on ligation of CD40 on DCs by CD40L expressed by activated CD4+ T-cells). Ligation of CD40 activates DCs, which then secrete cytokines such as IL-12, a vital third signal in priming of T-cell responses[234,287,288]. The original data regarding CASAC suggested that inclusion of either a Class II peptide or an agonistic anti-CD40 antibody in CASAC could effectively stimulate OVA-SIINF specific CD8+ T-cell expansion[238]. One benefit of this approach, should it be equally efficacious to vaccinations including a Class II peptide, would be to avoid the requirement to identify individual immunogenic Class II peptides within the target antigen binding to selected HLA Class II molecules.

To address whether anti-CD40 antibody could substitute for, or even promote more sizable and potent WT1-specific immune responses, anti-CD40 antibody was compared with OVA-ISQ or WT1-PGC in WT1-RMF and CASAC vaccinations (see Figure 4-17). This comparison demonstrated no significant differences with respect to short-term immune response generation where a related or unrelated Class II peptide or anti-CD40 antibody was used as part of CASAC. Overall, the magnitude of the observed immune responses in the WT1-RMF immunised mice was lower than that observed in earlier experiments. The vigorous responses seen in the OVA-SIINF immunised mice indicates that all CASAC components were functioning effectively, suggesting that the variability of observed responses against WT1 epitopes may be related to properties of this particular peptide and/or the difficulty of breaking tolerance to induce an effective immune response against a self-antigen.

Figure 4-17 Related or unrelated Class II peptides, or an agonist anti-CD40 antibody may be used in CASAC vaccinations to promote WT1-specific immune responses.





Figures 4-17a, b this page, 4-17c, following page. Mice (n=3-4) were immunised 4 times with CASAC and OVA-SIINF (plus either OVA-ISQ or anti-CD40 antibody) or CASAC and WT1-RMF (plus OVA-ISQ, anti-CD40 antibody or WT1-derived Class II peptide WT1-PGC). Figure 4-17a (this page): Frequencies of antigen-specific CD8+ T-cells following 3 rounds of vaccination were detected using OVA-SIINF/K^b or WT1-RMF/D^b pentamers in OVA-SIINF or WT1-RMF-immunised mice respectively. LCMV (FQPQ/D^b) irrelevant pentamer staining of representative PBMC samples from each group of immunised mice served as negative controls. Fb *In vivo* lysis of splenocytes loaded with the immunising peptide (OVA-SIINF or WT1-RMF) in OVA-SIINF and WT1-RMF immunised mice respectively, was measured following 4 rounds of vaccination. Unimmunised mice were challenged with OVA-SIINF loaded splenocytes (n=2), or WT1-RMF-loaded splenocytes (n=2). No statistically significant differences were detected between groups. Mean and SD per group are shown in each graph. Figures 4-17a, b this page, 4-17c, following page.

c) Frequencies of IFNγ secreting CD8+ T-cells following 4 rounds of CASAC vaccinations incorporating a related/unrelated helper peptide or agonist anti-CD40 antibody

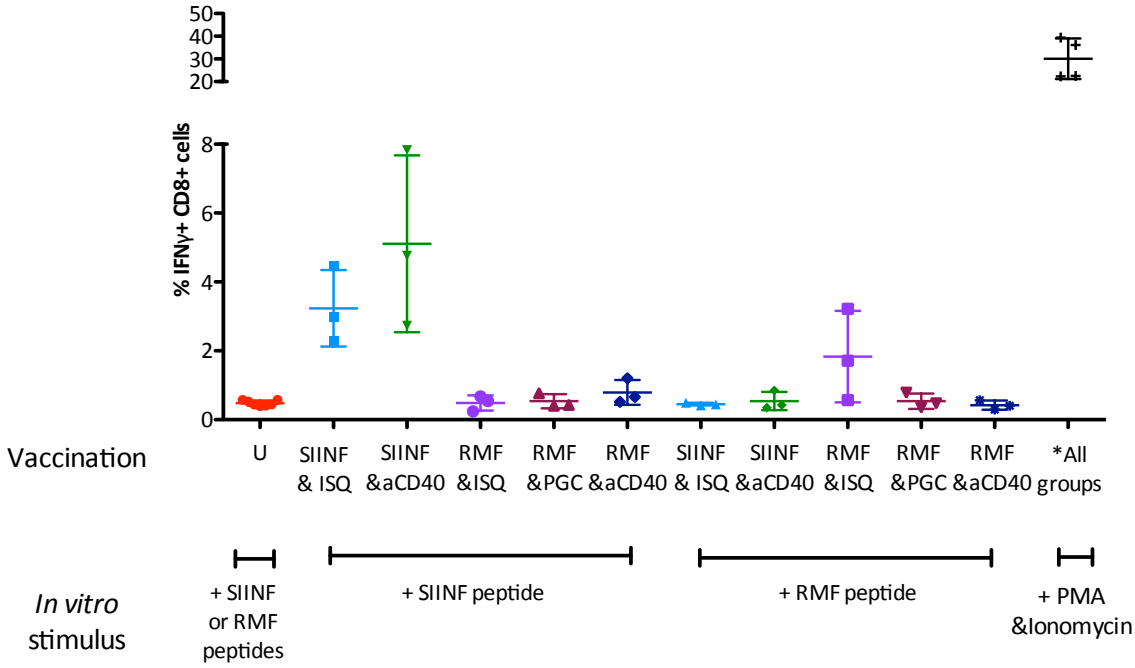


Figure 4-17 continued

(c) Antigen-specific production of IFNγ was assessed by stimulation of splenocytes harvested following 4 rounds of vaccination with relevant (immunising) or irrelevant, non-immunising peptides. No statistically significant differences were detected between groups. Mean and SD per group are shown. U, unimmunised; aCD40, agonist anti-CD40 antibody; PMA, Phorbol 12-myristate 13-acetate.

4.2.2.4 Summary

The results from these studies demonstrated the following:

1. The observation that increasing doses of WT1-RMF peptide did not increase the magnitude of WT1-RMF specific CD8+ T-cell frequencies or *in vivo* lysis contrasts with the published observations regarding TRP-$2_{180\text{-}188}$[238]. Although the cause for this is unknown, it suggests that increasing exposure to WT1-RMF dose during vaccination is insufficient to overcome tolerance mechanisms controlling responses against this particular self-antigen.
2. Another example of the differential behaviour of immunising peptides within the context of CASAC was the demonstration that alterations in the squalene composition of emulsion could affect the magnitude of WT1-RMF specific responses but not OVA-SIINF specific responses. This

observation highlights the need to assess each candidate peptide rigorously within the context of CASAC, as separate components of vaccination may need to be optimised to reach maximal efficacy for a given peptide.

3. Within the studies presented here, inclusion of a Class II peptide that is related, or unrelated, to the target Class I peptide, or agonist anti-CD40 antibody, increases the frequency of responders to WT1-RMF vaccinations using CASAC. However, no clear advantage for inclusion of helper epitope or agonist anti-CD40 antibody for short-term induction of WT1-specific immunity could be demonstrated due to within group variation. The utility of relevant/irrelevant helper epitopes or agonist anti-CD40 antibody in CASAC vaccinations is in keeping with the published data regarding CASAC vaccinations[238].

4.2.3 Extension of CASAC vaccinations targeting WT1 to the clinical context: immunisations using a WT1 peptide pool and comparison of CASAC vaccinations with complete Freund's adjuvant

The ultimate goal of these preclinical studies of CASAC and WT1 peptide vaccination is to identify a broad strategy, applicable to all potential patients irrespective of HLA type, which effectively induces WT1-specific cytotoxic T-cell responses. Such a vaccination must be at least as efficacious as the current best available strategies, described in the Introduction (section 1.6.2). Not only the choice of WT1 peptides but also the adjuvant components used for vaccinations are under investigation in this context. CASAC needs to show equivalence or superiority over currently used adjuvants (whilst maintaining safety and preferably increased tolerability) before it can be translated into the clinical trial setting. The following studies describe means to increase applicability of WT1 peptide vaccination to all patients (rather than those with selected HLA types) and to compare the efficacy of CASAC for vaccinations over the current standard vaccine adjuvant (Freund's adjuvant).

4.2.3.1 Use of a pool of overlapping WT1 peptides combined with CASAC to induce WT1-RMF specific immune responses

Whilst different Class II peptides have been shown in 4.2.3 to combine with CASAC vaccinations targeting WT1-RMF to induce WT1-RMF specific immune responses, no single Class II peptide was shown to be superior over the other with respect to ability to induce antigen specific immune responses in vaccinated

mice. The optimal WT1-derived helper peptide for use in vaccinations is unclear. Indeed it is possible that several potentially effective Class II helper peptides exist within WT1. This has been suggested by recent findings from Doubrovina et al[215].

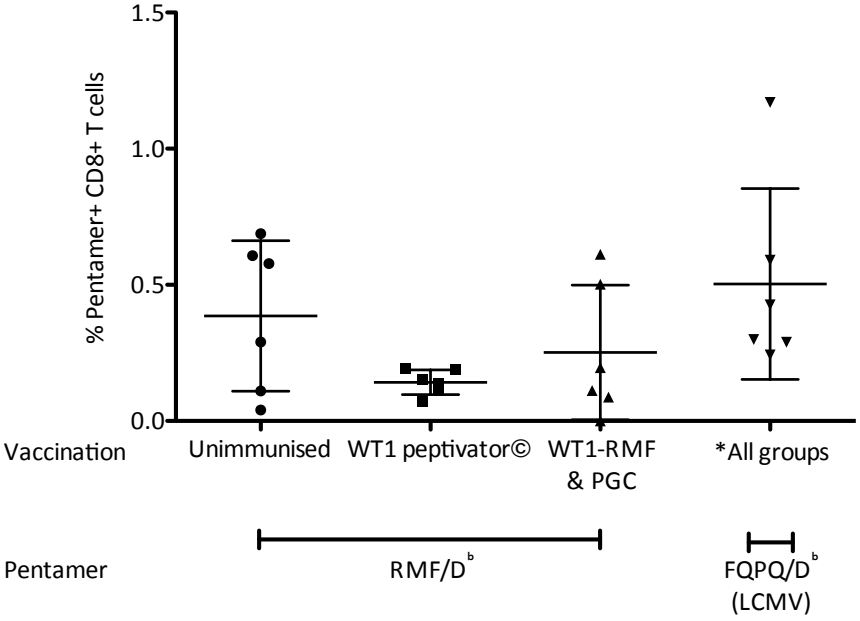
The overlapping peptide pool WT1 PepTivator® is therefore attractive as a means to potentially induce a range of responses against WT1 epitopes without requirement for matching to the individual HLA-types. The cocktail consists of 113 peptides, mainly 15-mer sequences, with 11 amino acid overlap, covering the entire sequence of the human WT1 protein. It was administered in CASAC vaccinations and given that the WT1-RMF epitope (one of the higher scoring peptides for binding to HLA-A*02:01/H-2D^{b}) can be detected using the WT1-RMF-specific pentamer, WT1-RMF specific immune response induction was used as a read-out. A dose approximating to 200μg of the WT1 peptide pool per immunization was compared with WT1-RMF along with WT1-PGC (amounting to a total dose of 200μg of peptide). Figure 4-18 demonstrates results following immunisations on days 1, 10, 21 and 28. There was no evidence (by pentamer staining) of an increase in WT1-RMF specific CD8+ T-cells in either of the immunized groups relative to unimmunized mice after 3 rounds of vaccination (Fig 4-18a). However, *in vivo* lysis of WT1-RMF loaded splenocytes after 4 vaccinations was observed in both immunized groups above that seen in the unimmunized controls. Within each group there was variability in the magnitude of the response. As observed in some of the earlier studies described in 4.2.1 and 4.2.2, there was a lack of correlation between detection of WT1-RMF specific T-cells by pentamer studies and functional activity as assessed by the *in vivo* lysis assay.

It is relevant that the quantity of the WT1-RMF epitope within the dose of WT1 PepTivator® administered in each vaccination is substantially smaller than that administered when the single WT1-RMF peptide was combined with WT1-PGC. The full RMFPNAPYL epitope is represented twice amongst the 113 overlapping WT1 sequences and therefore the quantity of WT1-RMF per vaccination with WT1 PepTivator® is approximately 4 μg, contrasting with the 100μg of WT1-RMF in each WT1-RMF and WT1-PGC vaccine. Despite this, equivalent *in vivo* lysis of WT1-RMF peptide loaded splenocytes was observed in both the WT1 peptide pool immunized mice and those mice immunized with the Class I and II peptides.

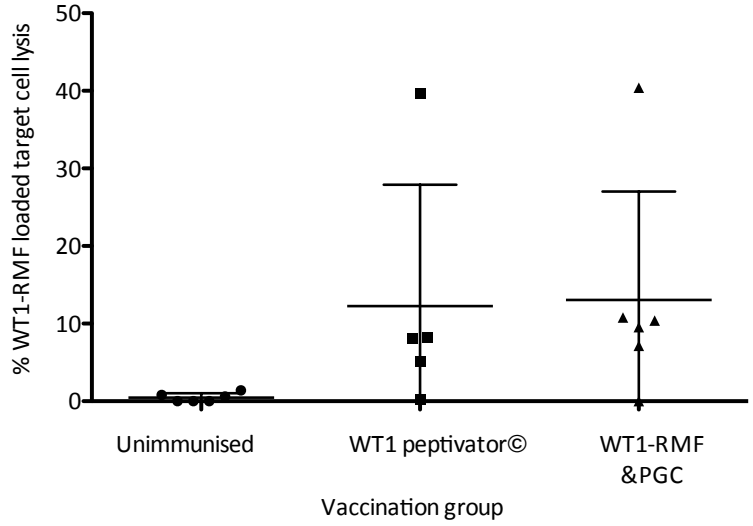
As such, it may be inferred that processing and cross-presentation of the WT1-RMF epitope derived from the peptide pool by DC's resulted in activation of responding CD8+ T-cells. In this study, this approach was at least as effective as immunizing with the individual Class I and II peptides at a higher concentration. The degree of *in vivo* lysis observed when using WT1-RMF and WT1-PGC was comparable to that observed previously (Figure 4-17) but overall appears lower than that seen using WT1-RMF alone for vaccination. On this occasion, WT1-RMF alone was not included as a condition but should be considered for inclusion in all future studies as a comparator since this has so far been the most successful for short-term induction of WT1-RMF specific immune responses. Induction of WT1-specific immune responses using the WT1 PepTivator® remains a tantalising area for future investigation and following optimisation would be amenable to clinical translation.

Figure 4-18 An overlapping peptide pool spanning the whole WT1 protein can combine with CASAC to induce WT1-RMF specific immune responses

a) Frequency of WT1-RMF specific CD8+ T-cells in WT1 peptivator immunised mice following 3 rounds of vaccination



b) *In vivo* lysis of WT1-RMF loaded target cells in WT1 peptivator immunised mice following 4 rounds of vaccination



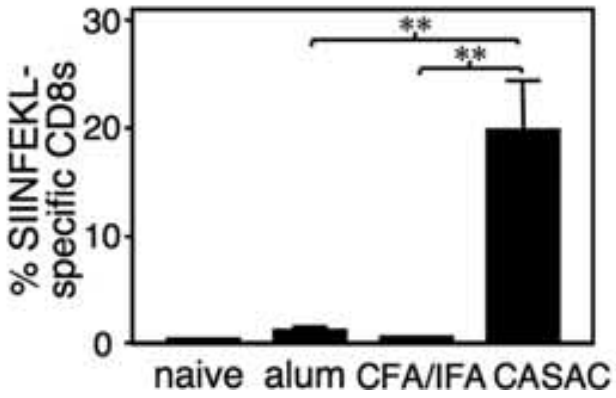
The efficacy of CASAC vaccination using the WT1 peptide pool versus 2 individual WT1-derived peptides (WT1-RMF & WT1-PGC) was assessed. (a) The frequency of WT1-RMF specific CD8+ T-cells following 3 rounds of vaccination are shown; representative samples from each group were stained with the irrelevant LCMV-specific FQPQ/D^b pentamer (*All groups). (b) *In vivo* lysis of WT1-RMF-loaded splenocytes following four rounds of vaccination. Mean and SD are shown for each group; no statistically significant differences were observed between groups for either of the 2 analyses. WT1 PepTivator®, overlapping WT1 peptide pool (Miltenyi Biotec Ltd).

4.2.3.2 Efficacy of CASAC vaccinations in comparison with current adjuvant systems: CASAC is as effective as complete Freund's adjuvant for induction of WT1-specific immune responses

A key issue in cancer vaccination is to identify potent mediators of cellular immunity that have minimal toxicity. Complete Freund's adjuvant (CFA) remains the standard adjuvant component used in pre-clinical vaccination studies. It consists of the mineral oil mannide monooleate containing killed *M.tuberculosis* organisms that trigger the immune response following uptake by macrophages or monocytes and therefore is an effective activator of cell-mediated immunity. However, CFA inclusion in vaccines causes granuloma formation and local irritation/inflammation at vaccination sites, such that it is not considered suitable for use in humans[289]. The mineral oil alone, lacking the mycobacterial component, is known as Incomplete Freund's Adjuvant (IFA) and is used for vaccinations after initial priming using CFA to avoid granulomatous lesion formation in experimental animals[289]. A clinically approved adjuvant based on the composition of IFA is Montanide[290], which has been used as a vaccine adjuvant in clinical trials of vaccinations, including WT1 peptide vaccination[199,201].

Wells used CFA/IFA as a comparator for studies of CASAC efficacy in the original publication and reported CASAC to be associated with a 20-30-fold higher expansion of OVA-SIINF-specific CD8+ T-cells in comparison with CFA/IFA after 2 rounds of vaccination (Figure 4-19).

Figure 4-19 CASAC vaccination induces significantly higher frequencies of OVA-SIINF specific CD8+ T-cells in comparison with Complete Freund's Adjuvant



Percentage of OVA-SIINF specific CD8+ T-cells assessed by pentamer studies after 2 rounds of immunisation using alum, CFA/IFA or CASAC as adjuvant (naïve unimmunised mice shown as controls). CFA, Complete Freund's Adjuvant; IFA, Incomplete Freund's Adjuvant.

Taken from Wells et al[238].

Furthermore, use of CASAC in immunisations containing the whole ovalbumin protein (OVA) enabled induction of an antibody response associated with a Th1-type of immune response. Rapid and sustained production of Th1-associated IgG2a antibodies at higher levels than IgG1 (an immunoglobulin subclass typically associated with Th2-type responses) was observed. This pattern of antibody responses was not seen with CFA/IFA or alum-based vaccinations[238].

Vaccinations targeting the WT1-RMF epitope using either CASAC or the CFA adjuvant system were therefore undertaken in order to allow a clinically relevant assessment of WT1 vaccinations using the novel adjuvant combination in direct comparison with the most well established experimental adjuvant. Four rounds of vaccination were used for this experiment, in contrast to that described by Wells et al[238]. In this experiment, CFA was used as the adjuvant for the first vaccination in the CFA-immunised group; 4.4% v/v squalene emulsion (with no TLR agonists or IFNγ) was used for the 3 booster vaccinations to allow a closer comparison with the emulsion used in the CASAC-immunised groups.

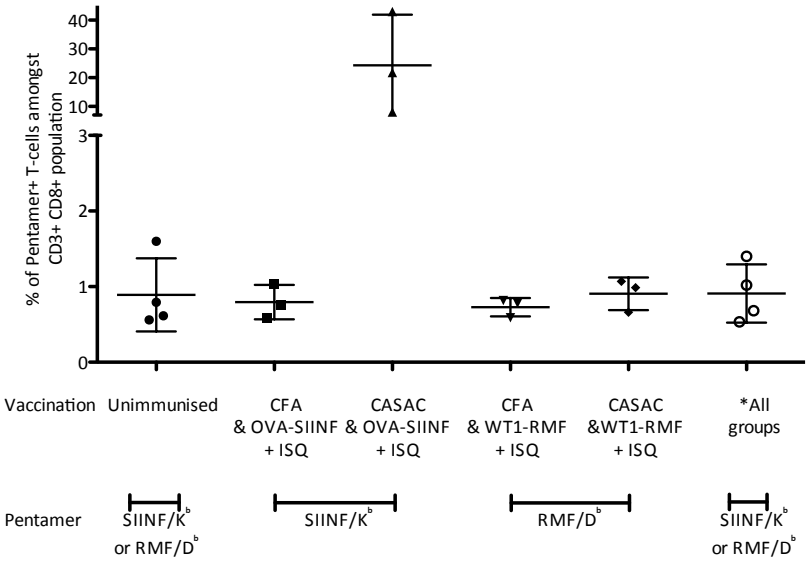
A significantly higher frequency of OVA-SIINF-specific CD8+ T-cells was observed following 3 rounds of vaccination using CASAC rather than CFA as the adjuvant, with no increase in pentamer staining above background in the CFA/OVA-SIINF immunised mice ($p<0.05$, Figure 4-20). This result is in keeping with the findings presented by Wells et al (shown in Figure 4-19)[238]. The *in vivo* lysis study however demonstrated that in the context of OVA-SIINF vaccinations, similarly potent *in vivo* cytolytic activity could be observed using CFA or CASAC as adjuvants (no statistically significant difference between the 2 conditions, Figure 4-20a). This is despite the lack of detection of OVA-SIINF-specific CD8+ T-cells induced using CFA/OVA-SIINF. This suggests that although the magnitude of the antigen-specific population induced by CFA/OVA-SIINF vaccination was small, those T-cells that had been primed were functionally effective. Also, it is possible that the use of the 4.4% v/v squalene emulsion rather than incomplete Freund's Adjuvant (IFA) for vaccinations 2, 3 and 4 may have produced more potent responses than would be seen using IFA (as described in Wells' data)[238]. An additional explanation for these findings is that in this study, 4 rounds of vaccination (rather than the 2 rounds described in the original publication) were used and could have sufficiently boosted even weaker immune responses induced by CFA and emulsion. This particular study

should be repeated in future using CFA for the first vaccination followed by IFA for subsequent immunisations 2, 3 and 4 versus CASAC.

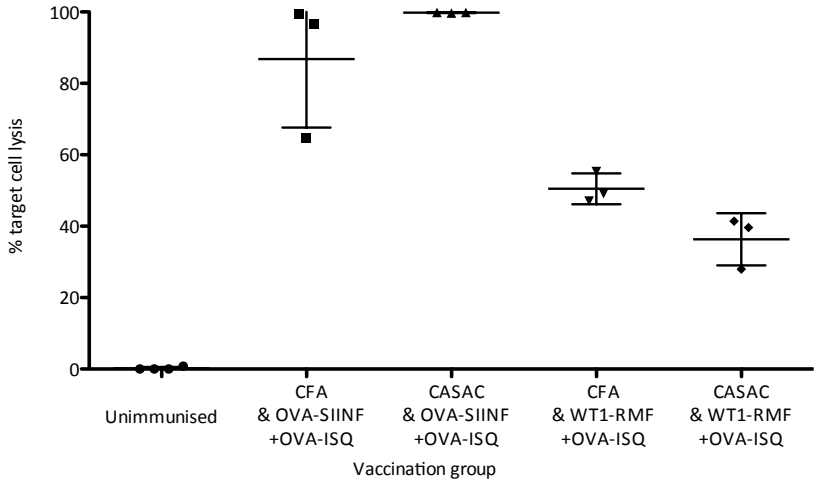
In the case of WT1-RMF immunisations, no increase in the frequencies of WT1-RMF-specific T-cells above background was demonstrated following 3 rounds of vaccination using CASAC or CFA as adjuvants (Figure 4-20a). As observed in the studies presented earlier, despite the absence of detectable WT1-RMF specific T-cells in these groups, large lytic responses were observed (Figure 4-20b). No significant differences were observed with respect to *in vivo* lysis of WT1-RMF loaded targets following WT1-RMF and CASAC or CFA immunisations. Wells et al did not describe a comparison of in vivo cytolysis following vaccinations against OVA-SIINF using CASAC or CFA as adjuvants in the original work[238]. The apparent equivalence of CASAC with CFA for induction of functional, WT1-RMF specific immune responses is encouraging, given that there has been no suitable, well-tolerated alternative to CFA for use in the clinical setting.

Figure 4-20 CASAC is as effective as Complete Freund's Adjuvant for promotion of WT1-RMF specific immune responses

a) Frequency of antigen-specific CD8+ T-cells following 3 rounds of vaccination using CASAC or CFA as adjuvants



b) *In vivo* lysis of target peptide-loaded splenocytes following 4 rounds of vaccination using CASAC or CFA as adjuvants



Results of immunisations using CFA or CASAC and WT1-RMF or OVA-SIINF with OVA-ISQ are shown. (a) Following 3 rounds of vaccination, OVA-SIINF/K^b and WT1-RMF/D^b pentamers were used to identify antigen-specific T-cell frequencies in OVA-SIINF or WT1-RMF-immunised mice respectively. *All groups: the pentamer containing the non-immunising peptide was used as a negative control in a sample from each immunised group. CD8+ T-cells from unimmunised mice were also stained with pentamers as controls. (b) In vivo lytic activity following immunisations was assessed using target splenocytes loaded with either OVA-SIINF or WT1-RMF peptide for OVA-SIINF or WT1-RMF-immunised mice respectively. The mean and SD per group are shown: no significant differences between groups were observed.

4.2.3.3 Summary

The results described in section 4.2.3 demonstrate the following:

1. That CASAC vaccination may combine with an overlapping peptide pool to induce WT1-RMF specific immune responses. Specifically, cross presentation of a Class I epitope within the WT1 peptide pool has taken place. It is conceivable that cross-presentation of other epitopes, binding to a range of Class I HLA molecules may also result from vaccination using these overlapping peptides. This could extend utility of WT1 peptide vaccination to patients with HLA types other than the most commonly studied alleles (such as HLA-A*02:01).
2. Repeated CASAC vaccination targeting WT1 is at least as effective as repeated vaccinations using CFA as the primary adjuvant. Further exploration of CASAC to combine with WT1 peptide vaccination should therefore be pursued, aiming to improve immune responses over and above those achieved with this “classical” adjuvant.

4.3 Discussion

The WT1 protein shows frequent over-expression by AML blasts and a range of solid tumours[291]. Accordingly, experts in immunotherapy have prioritized this protein for detailed evaluation as a therapeutic target using adoptive transfer of WT1-specific T-cells and immunization approaches[157]. Pre-clinical and clinical studies of WT1 peptide vaccination in the context of myeloid malignancies (described in chapter 1) have demonstrated evidence of vaccination-induced WT1-specific T-cell responses. In a few cases, such responses have coincided with disease stabilisation. Predominantly, immune responses have been of low magnitude and not sustained beyond treatment termination. Furthermore the majority of vaccination studies have focused on vaccinations using a handful of single epitopes (usually Class I) directed at specific HLA types, such as HLA-A*02:01[198-201]. There is therefore a clear need to improve the potency of WT1 vaccination as well as its applicability to all HLA types.

This chapter has described a novel approach, using a synergistic combination of adjuvants, to induce cell-mediated immune responses against WT1 epitopes. Repeated vaccinations using CASAC and WT1-RMF can result in large expansions of WT1-RMF specific CD8+ T-cells. The frequencies of WT1-RMF specific CD8+ T-cells induced after 3 or 4 vaccinations using CASAC were at least as large, and occasionally much greater, than those described by Kohrt et al. in the most comparable study of WT1-RMF peptide vaccination in healthy C57BL/6 mice[182]. Following 4 WT1-RMF peptide vaccinations administered in IFA, this group observed ~1% WT1-RMF specific cells amongst CD8+ T-cells using WT1-RMF tetramer analyses, shown in Figure 1-5. This group did not specifically assess lysis of WT1-RMF loaded targets. However, after short term cultures of lymphocytes isolated from mice following 4 vaccinations with a WT1-expressing tumour cell line, IFNγ production reached 10-13% by CD8+ T-cells. The frequency of WT1-RMF specific T-cells amongst the CD8+ T-cell population induced using CASAC reached up to 10-30% in some of our presented studies. These WT1-specific T-cells were also functional, effectively lysing WT1-RMF loaded target cells *in vivo* and secreting IFNγ (at best, up to 15-20% of CD8+ T-cells in responding mice) following re-stimulation with WT1-RMF peptide *in vitro*.

Similarly to Man, the WT1 protein shows a tissue-restricted pattern of expression throughout embryonic development and into adulthood in mice[74]. Therefore the ability to induce WT1-specific T-cell responses following repeated vaccination suggests that tolerance mechanisms have been subverted. Despite this, no mice immunized with WT1 peptide(s) died or showed loss of condition during the course of therapy, suggesting no autoimmune toxicity. CASAC was at least as effective at inducing WT1-RMF specific immune responses as the most commonly used pre-clinical adjuvant, Complete Freund's Adjuvant (CFA). Given the limited range of clinically acceptable adjuvants for cell-mediated immunity, the presented findings provide a strong basis for future investigation of CASAC and WT1 peptide vaccinations for AML therapy.

Wells et al had documented the ability of CASAC to combine with whole protein (ovalbumin) immunisations and result in cross-presentation of the OVA-SIINF epitope to cognate CD8+ T-cells[238]. Indeed, ligation of TLR3[292] and TLR9[293,294] has been shown to promote cross-presentation of endocytosed antigens by DCs. These observations provided a rationale for exploring CASAC in combination with the WT1 PepTivator® for immunisations, since the long peptides (15mers) would need to be cross-presented in order to elicit cytotoxic T-cell responses. Such an approach, if shown to be efficacious, would be attractive, due to the potential to broaden the applicability of WT1 peptide vaccination to a range of HLA types.

The demonstration of CD8+ T-cells recognising the WT1-RMF epitope following 4 rounds of immunisations using the overlapping WT1 peptide pool, despite the considerably lower concentration of the WT1-RMF epitope within the dose of WT1 PepTivator® administered per mouse, is encouraging. No significant differences in the frequencies of WT1-RMF specific CD8+ T-cells or magnitude of WT1+ target cell lysis were detected between the group receiving WT1 PepTivator® or WT1-RMF and WT1-PGC in immunisations. These observations suggest that long peptides within WT1 PepTivator® can be processed effectively to allow cross-presentation of WT1-RMF by professional APCs in the context of H-2D^{b} to cognate CD8+ T-cells. This parallels observations made *in vitro* by Doubrovina et al, whereby healthy donor PBMCs were cultured with an overlapping peptide pool encompassing the whole of WT1, eliciting CD4+ and CD8+ T-cell responses[215]. The WT1-RMF epitope nested within the pool was cross-presented to

CD8+ CTLs, resulting in IFNγ production and lysis of primary WT1+ leukaemic targets[215]. Significantly, WT1-RMF was found to be just one of 42 immunogenic epitopes within the WT1 peptide pool, most of which were previously unknown. In fact WT1-RMF elicited T-cell responses of smaller magnitude than some of the other newly identified epitopes. Furthermore, 10 of the novel epitopes could activate T-cells from donors of up to 4 different HLA-types[215]. Our studies concentrated on the ability to use the WT1 PepTivator® to immunise mice, using the identification of WT1-RMF specific T-cell responses as a read-out, due to well-established immunogenicity of this epitope and availability of WT1-RMF pentamers. However, the complementary findings of Doubrovina et al highlight the potential for the WT1 PepTivator® to generate immune responses within donors (and patients), some of which may be more potent than those induced by the most well-studied WT1 epitope to date, WT1-RMF[215]. Given the observation by Rezvani et al that repeated vaccination targeting the WT1-RMF epitope alone induced predominantly low avidity CTL responses[200], our findings in combination with those of Doubrovina et al support exploration of the role of WT1 PepTivator®/CASAC immunisations in future studies.

I also demonstrated that certain peptides predicted to combine with CASAC to enhance promotion of WT1-RMF specific immune responses did not achieve the expected results. Firstly, the heteroclitic peptide WT1-YMF was not superior to WT1-RMF for induction of WT1-RMF specific cytotoxic or IFNγ responses using CASAC in C57BL/6 mice. This contrasted with findings in humans, where *in vitro* studies (such as measurement of IFNγ production upon challenge with WT1+ targets by T-cell lines derived from prolonged cultures with WT1-RMF or WT1-YMF) suggested that the heteroclitic rather than native peptide might more efficaciously induce T-cell responses against the native peptide. This had been predicted based on higher binding affinity of WT1-YMF for HLA A*02:01 in comparison with WT1-RMF (SYFPEITHI scores 24 and 22 respectively). As shown in Table 4-1, higher binding affinity and lower IC_{50} concentrations for WT1-YMF over WT1-RMF in the context of H-2D^b suggested this might also be the case in mice. However significantly higher *in vivo* lysis of the WT1-RMF loaded targets in mice immunised with the native rather than the heteroclitic peptide was found (Figure 4-8). Secretion of IFNγ was also higher upon *in vitro* re-challenge with WT1-RMF for mice immunised with WT1-RMF rather than WT1-YMF. Results therefore show that WT1-YMF is not effective as a heteroclitic modification in the context of murine H-2D^b. It is also possible that the interaction between D^b/YMF and the murine TCR is less

favourable than that with D^b/RMF. This has been questioned in the context of human TCR/HLA A*02:01 interactions, following a recent study of the crystallographic structure of the human HLA-A*02:01/WT1-YMF complex interacting with the human WT1-TCR. Borbulevych et al. demonstrated that the WT1-YMF peptide bound to the HLA-A*02:01 binding groove in similar configuration to WT1-RMF. However, the WT1-YMF peptide altered the position of charged side chains and the electrostatic surface potential of the HLA-A*02:01/peptide complex when compared to the WT1-RMF peptide/HLA complex. The authors concluded that such alterations may negatively impact on the WT1-TCR recognition of the WT1-YMF/HLA A*02:01 complex, given the recognised role of side chain interactions within the TCR-MHC/peptide complex in determining binding affinity[295]. My findings highlight caveats in extrapolating immune response induction in mice and humans and the need to verify *in silico* predictions experimentally. Furthermore, given reports suggesting that heteroclitic peptides do not always induce immune responses that recognise the native peptides and can show different results when evaluated *in vivo* versus *in vitro*[206,296], heteroclitic epitopes should be thoroughly evaluated before clinical translation.

Secondly, vaccinations combining CASAC with the universal Class II helper epitope PADRE were less efficacious for expansion of functional WT1-RMF or OVA-SIINF specific CD8+ T-cells than using the Class I peptide alone. The reasons for these observations are not known. PADRE itself had not been previously assessed as a helper peptide in the context of CASAC. Whilst there are different sequences of "PADRE" peptide with differential "helper" potencies, the particular PADRE sequence used in our immunisations had been shown to be efficacious as a helper epitope when administered in saline to C57BL/6 mice[266]. In that study however, a single vaccination was administered with the assessments of helper activity determined following subsequent *in vitro* culture[266]. PADRE was administered with WT1-RMF in IFA as a comparator group in a study of WT1 DNA vaccination: IFNγ production using ELISpot analysis showed no advantage for combining PADRE with WT1-RMF to induce functional CTL responses[297]. However, other studies, using PADRE fused to the target Class I epitope either in peptide or DNA vaccinations against viral or tumour antigens have suggested efficacy of this universal helper epitope[284,298]. One recent pilot study of a multi-epitope vaccine in humans (including WT1-RMF as one of the peptides and using Montanide as the oil-based adjuvant) demonstrated an increase in PADRE specific T-helper cells after vaccination. However, these CD4+ T-cells produced limited IL-2 and overall an increase in T-regulatory cells was

observed[299]. Although T-regulatory cells were not observed in our studies to have increased in the peripheral blood and in fact appeared to be lower in mice that received PADRE immunisations, the possibility that T-regulatory cells expanded at the draining lymph node sites cannot be excluded. Ultimately, the decision to evaluate the potential for PADRE to assist in WT1-RMF vaccination was based on having a universal helper epitope to broaden the applicability of WT1-RMF and CASAC vaccinations: given the potential immunogenicity of WT1 PepTivator®, the role of PADRE is somewhat redundant.

The findings above highlight the importance of thoroughly evaluating individual peptides in the context of CASAC vaccinations. Wells et al had demonstrated previously that CASAC combines with variable efficacy with individual peptides to induce antigen-specific immune responses during their examination of a selection of self and foreign Class I peptides for induction of antigen-specific CD8+ T-cell expansion[238]. Clearly induction of responses against a self-antigen requires subversion of tolerance mechanisms that do not govern the development of responses against a xenoantigen, such as OVA-SIINF. However, of 4 self-peptides evaluated by Wells et al in CASAC vaccinations, only 3 led to CD8+ T-cell expansion. One explanation for the difference in immunogenicity of these peptides was the lower SYFPEITHI binding scores of the poorly immunogenic self-peptides[238]. My findings also suggest that other factors, such as emulsion composition, may need to be optimised and tailored to the individual immunising peptide, since alteration of the squalene percentage within the emulsion influenced the magnitude of the WT1-RMF specific immune responses generated. In concordance with the previous studies of CASAC, alteration of the squalene quantity within the emulsion when targeting the immunogenic foreign epitope OVA-SIINF did not affect immune response induction. The observation that 4.4% v/v squalene emulsion, but not 2.2% or 8% squalene v/v emulsion could induce WT1-RMF specific CD8+ T-cell responses suggests that induction of T-cell responses against this self antigen are finely balanced and small differences in the vaccine composition can affect outcomes. The greater ease of generating an immune response against a foreign epitope may explain why such apparently minor variations have a lesser impact on immune response induction.

The difficulty of stimulating immune responses against WT1-RMF was also highlighted by the variability of results, both within groups of mice and between experiments. This variability was present in all experiments and consistency was not improved by modulation of CASAC components or escalation of the immunising peptide dose in our studies. Despite using the same mouse strain and fresh stocks of reagents from consistent suppliers, the frequency of WT1-RMF specific CD8+ T-cells could range from below 1% to almost 30% and the degree of lysis of WT1-RMF loaded targets ranged from <10% to approximately 80%. Indeed in some vaccination experiments, no WT1-RMF specific immune responses (evaluated by pentamer or *in vivo* lysis studies) could be observed after 4 rounds of vaccination (data not shown). In 2 of these studies, no WT1-RMF-specific T-cell expansion or lysis of WT1-RMF loaded targets immune responses could be induced using either WT1-RMF or the WT1 PepTivator®. In these same experiments, CASAC would consistently promote OVA-SIINF specific responses of the expected magnitude based on the work of Wells and our own observations[238]. CASAC and OVA-SIINF immunised mice were considered as positive controls for vaccination since the observed large magnitude, functionally highly potent immune responses induced suggested integrity of the CASAC components themselves. No changes were made to the handling and storage of the WT1-RMF peptide and the same supplier of synthetic peptides was used throughout the study. The ability of thawed WT1-RMF to stabilise HLA-A*02:01 molecules on the surface of T2 cells was used as a surrogate to suggest integrity of the WT1-RMF peptide following freezing and thawing. Despite this, no clear cause for the variability of immune responses between mice was found during the time-period available for these studies. It is most likely that this observation reflects the need to consider other factors that may combine with CASAC to influence immune response generation. This could include the addition of even more stimulatory signals, such as IFNα, anti-CD40 ligation in combination with Class II peptide provision or a third TLR agonist. Removal of inhibitory factors, such as regulatory T-cells or signals e.g., via CTLA-4, PD1/PDL1 could help to tip the balance in favour of WT1-RMF specific T-cell activation and reduce variability in the observed immune responses. These suggestions will be explored further in Chapter 6.

In conclusion, the use of CASAC, particularly in combination with the WT1 overlapping peptide pool, has shown potential for effective induction of WT1-specific immune responses that could be applied across the HLA spectrum. Future work should aim to optimise these immune responses further, to reduce

variability in their magnitude and potency, prior to assessment of therapeutic benefit in a murine leukaemia model. Should anti-leukaemic responses be observed pre-clinically, an attractive therapeutic approach would be to use CASAC/WT1 peptide vaccination in AML patients with WT1-over expressing blasts for remission maintenance following debulking chemotherapy, with the ultimate aim of eradicating WT1-expressing leukaemic stem cells.

Chapter 5 Whole cell vaccination using CD80/IL-2 modified AML blasts for remission maintenance in patients with relapsed AML post allogeneic HSCT.

5.1 Introduction

Chapter 3 described that patients with relapsed AML and MDS post allogeneic HSCT show an estimated 5-year overall survival of 40% following treatment with therapeutic DLI (tDLI). However, the 5-year relapse/progression rate was 69% (54%-81%, CI 95%), with only a minority of patients experiencing durable remissions following tDLI. In the majority of patients analysed, there was either no response to tDLI or leukaemia ultimately recurred, highlighting that induction of GvL activity was neither universal nor sustained in this setting. Incidence of GvHD was still moderate, occurring in 41% of patients post-tDLI and was more commonly observed in those who experienced a disease response to tDLI. Whilst these findings support the therapeutic potential of DLI, there is a clear need to enhance the efficacy of this treatment to increase response rates overall and confer sustained GvL activity. Strategies that can favour induction of effective GvL provision by DLI whilst limiting GvHD induction are highly desirable.

Accumulating evidence supports the crucial role of activation of naïve alloreactive donor T-cells in providing GvL activity post-HSCT[42,300,301]. It is likely that minor histocompatibility antigen (mHag)-derived peptides, in the context of a fully HLA-matched allo-HSCT, will provide the most potent stimuli to activate these naïve T-cells because these epitopes will be entirely novel to the donor immune system[42,301]. Indeed, T cells that recognize haematopoietic cell-restricted, mHag-derived epitopes from DLI-responsive patients have been isolated[300,302-304]. Tissue restricted expression of these mHags to cells of the haematopoietic system suggests that induction of responses against these targets may result in GvL without damage to other organs (GvHD). Furthermore, correlation of the emergence of leukaemia antigen-specific T-cells in recipients following DLI with disease control suggests that these epitopes may also be able to induce GvL activity[305,306]. In one study, low frequencies of WT1-specific donor T-cells were detectable in the pre-infusion DLI products; following infusion and isolation of T-cells from PB of the recipients, expansion of the same clones and additional, newly arising, WT1-specific clones were identified *in vitro*[305]. Graft-versus-leukaemia responses may include not only CD8+ T-cell but also CD4+ T-

cell responses: Rutten et al have recently isolated alloreactive CD4+ T-cells detectable after DLI in responding patients and specific for patient, but not donor, HLA-DP molecules[307]. Since the expression of HLA Class II molecules is largely confined to haematopoietic cells, this provides an additional target for leukaemia-specific responses. It is therefore plausible that active approaches to ensure effective stimulation of infused naïve donor CD4+ and CD8+ T-cells against a range of leukaemia-associated or specific antigens will increase the efficacy of this treatment. Targeting several antigens, rather than a single antigen, may avoid immunoselection pressures favouring the generation of tumour escape mutants.

The variable response rates observed in studies of therapeutic DLI suggest that, despite provision of immune competent cells in the infused product capable of GvL activity, the patients' leukaemic cells may fail to encounter or effectively stimulate these cells. As outlined in Chapter 1, not only do AML blasts express a range of target leukaemia-associated antigens (see Table 1-1)[46] and potentially donor-recipient discordant mHags[59,300,304], but they also express MHC Class I and II antigens and often adhesion molecules including ICAM1[99,308,309]. These features should enable them to act effectively as APCs. However, they also show low expression of the vital co-stimulatory molecule CD80 and may secrete immunosuppressive factors[99,308,309]. AML blasts are therefore likely to be sub-optimal stimulators of immune responses. Importantly, ineffective co-stimulation of responding naïve T-cells results in induction of an anergic state and failure to respond to their target[310].

Gene therapy affords an opportunity to overcome these deficiencies and boost immune cell activation by AML blasts. Expression of CD80 by genetically engineered AML blasts may increase their ability to co-stimulate naïve T-cells. Induction of IL-2 secretion by gene transfer provides a proliferative stimulus to lymphocytes and may reverse an anergic state[310]. As described in Chapter 1, *in vitro* and *in vivo* data have demonstrated the superior induction of leukaemia-specific cytolytic activity in responder T-cells by CD80/IL-2 modified AML blasts compared with activation by unmodified AML blasts or blasts modified to express CD80 or IL-2 alone[120,258]. Allogeneic T-cells previously co-cultured with CD80/IL-2 modified AML blasts demonstrated significant IFNγ production and lysis of primary unmodified AML blasts on

subsequent challenge, despite the presence of immune suppressive Tregs in some cultures[261]. Furthermore, the enhanced ability of CD80/IL-2 modified AML blasts to activate NK-cell mediated cytotoxicity provides an additional means to stimulate leukaemic cell lysis[121]. Prophylactic, single gene-modified AML cell vaccination within the first 100 days post allo-HSCT for high risk AML/MDS has been reported[246]. However, the novel approach described here combines vaccinations comprising tumour cells modified to express two complementary immunostimulatory genes, with provision of an immune competent donor repertoire in the form of donor lymphocyte infusions, in order to generate a more effective GvL response.

Herein, a phase I study to assess safety and toxicity of CD80/IL-2 gene-modified AML cell vaccination in patients with relapsed AML post-allogeneic HSCT currently recruiting at King's College Hospital is reported. Following morphological remission re-induction using salvage chemotherapy, four patients have been treated to date. Two subjects have been allocated to each arm (Arm A: standard care with escalating dose DLI vs. Arm B: treatment with escalating dose DLI co-administered with the patient-specific, gene-modified, CD80/IL-2 expressing AML Cell Vaccine, termed ACV for ease of reference). The data presented in this chapter demonstrate that vaccination of two patients has been clinically safe. There has been no evidence of replication competent lentivirus formation as a result of gene therapy, or systemic elevation of IL-2 due to vaccination. Ability to respond to vaccination will be dependent on immune competence. Therefore, the numbers and relative frequencies of lymphocyte subsets in peripheral blood of subjects during treatment and follow up were assessed and compared to healthy age-matched volunteers.

5.2 Results

5.2.1 Characteristics of patients screened for potential trial entry

Between 1st July 2009 and 28th February 2013 inclusive, a total of 41 patients underwent AML blast collection either at the time of AML diagnosis or at relapse post-HSCT (Table 5-1). In 2009, collection was performed for one patient and 12, 16, 10, and 2 were screened in 2010, 2011, 2012 and until March 2013 respectively. Peripheral blood and bone marrow mononuclear cells (PBMC and BMMC respectively) were cryopreserved under GMP conditions for all eligible patients. Blood and bone marrow harvest yielded

sufficient viable cells to permit production of a minimum of 3 doses of the AML Cell Vaccine (ACV) in 36 of the screened patients. Four of these patients were eventually enrolled onto the study.

Reasons for non-enrolment included:

(1) patient did not undergo HSCT (n=6),

(2) persistent disease following chemotherapy for relapse post-HSCT and/or following chemotherapy (n=11),

(3) presence of exclusion criteria (active GvHD, n=4; presence of comorbidity, n=1; primary diagnosis of myelodysplastic syndrome with excess of blasts rather than AML, n=1; patients whose cells were stored at diagnosis did not relapse post-HSCT, n=3) or

(4) inability to locate donor to obtain donor lymphocytes (n=1).

Four patients were continuing treatment for relapse as of March 2013.

Two eligible patients (UPN 31 and UPN 38) could not be enrolled onto the study as the ACV failed to meet specifications permitting product release on trial. The specifications are:

1. CD80 expression: ≥20% of cells in the vaccine product must express CD80 with at least a 5-fold increase in MFI above cells stained with an isotype control in place of anti-CD80
2. IL-2 secretion: $\geq$ 0.1ng/10^6 cells and $\leq$ 25 ng/10^6 cells per 24 hours
3. Viability $\geq$ 50% based on Annexin-V staining, prior to freezing
4. Sufficient cell numbers to achieve the minimum 3 doses of ACV (Dose 1: 0.5-1x10^5 cells, Dose 2: 0.5-1x10^6 cells, Dose 3: 0.5-1x10^7 cells, Dose 4: 0.5-1x10^8 cells).

CD80 was expressed by only 8.5% of cells with just a 2.05-fold increase in MFI above the control population in the product manufactured for UPN 31. Furthermore no IL-2 secretion was detected. For UPN 38, 22% of cells expressed CD80 (associated with a 4.19-fold increase in MFI). IL-2 secretion was low, at 0.035 ng/10^6 cells per 24 hours.

Variability in the transduction efficacy of primary AML blasts has been observed in prior pre-clinical *in vitro* studies[258] suggesting that AML cells may possess inherently different susceptibility to infection with the lentiviral vector. No specific causes for the UPN31 and UPN38 transduction failures were identified. Transduction of U937 targets performed in parallel was successful in both cases indicating that a procedural fault was unlikely. Myeloid blasts comprised 56% and 23% of the total nucleated cells in the bone marrow of UPN 31 and UPN 38 respectively, and showed typical myeloid phenotype (CD34+, CD13+, CD117+ with myeloperoxidase expression) and karyotypes (normal in UPN 31 and inversion 16 in UPN 38). Therefore adequate numbers of AML blasts with typical surface marker expression were present in the BMMCs used in lentiviral transduction cultures. However, cell viability was <50% for the product produced for UPN 31 and 58% (just over the specification threshold) for UPN 38. Therefore low cell viability may have contributed to transduction failures. Ongoing investigations to refine the transduction procedure seek to increase the efficacy of this process.

Table 5-1 Characteristics of patients undergoing AML blast cryopreservation

Unique Patient number (UPN)	Patient age at screening & sex	HSCT conditioning & donor type	Screening time-point	Time from HSCT to harvest, days	BM blast % at relapse by morphology	Donor CD3% at relapse	Cell harvest yield (viable cells)		Salvage chemotherapy received for relapse post-HSCT	Response to salvage chemo-therapy	Enrolment or reason for non-enrolment
							PBMC	BMMC			
UPN1	58, F	N/A	Diagnosis	N/A	18%	N/A	$3.4x10^8$	Nil	N/A	N/A	No HSCT
UPN2	58, M	FBC, (S)	R1, post-HSCT	N/A	46%	9%	$2.3x10^8$	Nil	N/A	N/A	Died of graft failure post-HSCT
UPN3	52, M	FBATG, (S)	Diagnosis	N/A	53%	N/A	Not banked	Not banked	N/A	N/A	Insufficient harvest
UPN4	42, F	N/A	Diagnosis	N/A	25%	N/A	$1.13x10^8$	Nil	N/A	N/A	No HSCT
UPN5	44, F	N/A	Diagnosis	N/A	90%	N/A	$1.1x10^9$	Nil	N/A	N/A	No HSCT
UPN6	54, M	FBC (V)	Diagnosis	N/A	91%	N/A	$1.9x10^9$	$5.8x10^8$	N/A	N/A	No HSCT
UPN7	55, M	FLAMSA (S)	R1, pre 2^{nd} HSCT (FBC)	260	40%	96%	$4.8x10^8$	*	N/A	N/A	No relapse post-HSCT
UPN8	65, M	N/A	R1, pre-HSCT	N/A	15%	N/A	Nil	$2.6x10^8$	N/A	N/A	No HSCT
UPN9	65, M	N/A	R1, pre-HSCT	N/A	21%	N/A	Not banked	Not banked	N/A	N/A	Insufficient harvest (and no HSCT)
UPN10	52, M	N/A	R1, pre-HSCT	N/A	67%	N/A	$1.6x10^9$	$1.6x10^8$	N/A	N/A	No HSCT
UPN11	60, M	FCTBI (H)	Diagnosis	N/A	28%	N/A	$4.8x10^8$	$4x10^8$	N/A	N/A	No relapse post-HSCT
UPN12	59, M	FBC (V)	R2, post-HSCT	308	40%	80%	$1.3x10^7$	$2.6x10^8$	FLAG	MR	Excluded: GvHD liver
UPN13	36, M	FB4C (V)	R1, post-HSCT	184	20%	56%	$4.1x10^7$	$6.5x10^7$	FLAG	MR, Cy+	Enrolled
UPN14	44, F	FBATG (S)	R1, post-HSCT	546	10%		Nil	$2.5x10^8$	FLAG	MR	Enrolled

* Combined cell count for PBMC and BMMC harvested given in PBMC column

Unique Patient number (UPN)	Patient age at screening & sex	HSCT conditioning & donor type	Screening time-point	Time from HSCT to harvest, days	BM blast % at relapse by morphology	Donor CD3% at relapse	Cell harvest yield (viable cells)		Salvage chemotherapy received for relapse post-HSCT	Response to salvage chemo-therapy	Enrolment or reason for non-enrolment
							PBMC	BMMC			
UPN15	64, M	FB4ATG (S)	R1, post-HSCT	98	85%	83%	$3.9x10^8$	$2.5x10^8$	FLAG	PD	Refractory AML
UPN16	67, M	FBC (V)	R1, post-HSCT	58	63%	93%	$8x10^7$	$3.7x10^7$	FLAG	PD	Died following chemotherapy
UPN17	61, F	FBC (V)	R1, post-HSCT	116	35%	56%	Not banked	Not banked	FLAG	PD	Insufficient harvest
UPN18	60, M	FBC (V)	R1, post-HSCT	178	8%	100%	$1.6x10^7$	$3.3x10^7$	FLAG	PD	Refractory AML
UPN19	68, M	FBC (V)	R1, post-HSCT	97	14%	100%	$3.8x10^7$	$7.8x10^7$	Died pre-chemotherapy	N/A	Died pre-chemotherapy
UPN20	35,F	FBC (V)	R1, post-HSCT	187	90%	98%	$2x10^7$	$1.7x10^8$	LD cytarabine	PD	Refractory AML
UPN21	38,M	FBATG (S)	R1, post-HSCT	533	5%	87%	$1.9x10^8$	$5.4x10^8$	5-Azacytidine	MR	Not eligible (primary diagnosis of MDS RAEB, not AML)
UPN22	52, F	FBATG (S)	R1, post-HSCT	1051	15%	86%	$8.4x10^7$	$9.4x10^7$	FLAG	MR	Enrolled
UPN23	62, F	FBATG (S)	R1, post-HSCT	335	24%	78%	$1.6x10^9$	$1.7x10^9$	5-Azacytidine	PD	Refractory AML
UPN24	36, F	Bu/Cy (S)	R1, post-HSCT	117	95%	100%	$1.4x10^8$	$8.1x10^8$	LD cytarabine, decitabine	MR	Not eligible (active GvHD)
UPN25	36, F	Bu/Cy (V)	R2, post-HSCT	404	22%	84%	Not banked	Not banked	FLAG	PD	Insufficient harvest
UPN26	40, M	FB4C (V)	R1, post-HSCT	466	9%	100%	Not banked	Not banked	FLAG	MR	Insufficient harvest
UPN27	63, M	FBC (V)	R2, post-HSCT	3021	90%	100%	$5.7x10^7$	$1.7x10^8$	FLAG, MiDAC	MR	Donor unavailable
UPN28	22, F	FCTBI (H)	R1, post-HSCT	100	76%	99%	$3.2x10^8$	$8x10^8$	Palliated	N/A	Refractory AML

Table 5-1 continued

Full details of transplant regimens are given in Appendix B. MR, morphological response; PD, progressive disease

Unique Patient number (UPN)	Patient age at screening & sex	HSCT conditioning & donor type	Screening time-point	Time from HSCT to harvest, days	BM blast % at relapse by morphology	Donor CD3% at relapse	Cell harvest yield (viable cells)		Salvage chemotherapy received for relapse post-HSCT	Response to salvage chemo-therapy	Enrolment or reason for non-enrolment
							PBMC	BMMC			
UPN29	60, M	FBC (V)	R2, post-HSCT	1460	27%	99%	$4.1x10^7$	$5.3x10^7$	FLAG	MR	Excluded (comorbidity)
UPN30	42, F	FLAMSA (V)	R1, post-HSCT	103	26%	97%	$1.7x10^8$	$6x10^7$	5-Azacytidine	MR	Excluded (active GvHD)
UPN31	71, F	FBC (V)	R1, post-HSCT	1881	23%	96%	$7.9x10^7$	$3x10^7$	Decitabine	MR	Failed vaccine production
UPN32	55, M	FB4ATG (S)	R1, post-HSCT	139	46%	83%	$5.2x10^7$	$5.2x10^7$	FLAG-Ida	MR	Enrolled
UPN33	66, M	FB4C (V)	R1, post-HSCT	54	39%	74%	$2.3x10^7$	$4.7x10^7$	5-Azacytidine	PD	Refractory AML
UPN34	60, F	FB4ATG (S)	R2, post-HSCT	188	63%	99%	$1.2x10^7$	$7.2x10^7$	FLAG-Ida	MR	Excluded (active GvHD)
UPN35	53, M	FB4ATG (S)	R1, post-HSCT	66	12%	79%	$4.7x10^8$	$2.2x10^8$	5-Azacytidine	Died during chemotherapy	Died
UPN36	50, M	FLAMSA (V)	R1, post-HSCT	142	16%	68%	$1.1x10^8$	$4.1x10^8$	FLAG-Ida, Decitabine	PD	Awaiting response
UPN37	65, M	FLAMSA	R1, post-HSCT	60	66%	98%	$9.3x10^7$	$4.5x10^8$	FLAG-Ida	Died during chemotherapy	Died
UPN38	50, M	FB4C (V)	R1, post-HSCT	349	63%	89%	$8.8x10^7$	$1.4x10^9$	HiDAC	MR, CCyR, Mol+	Failed vaccine production
UPN39	43, M	FB4C (V)	R1, post-HSCT	180	38%	77%	$5.4x10^7$	$8.9x10^6$	FLAG-Ida	PD	Awaiting response
UPN40	67, M	N/A	R1, pre-HSCT	N/A	35%	N/A	$1.4x10^8$	$1.9x10^8$	N/A	N/A	Await transplant
UPN41	67, F	FBATG (S)	R2, post-HSCT	2015	6%	99%	$4x10^8$	$3.3x10^8$	5-Azacytidine	Treatment ongoing	Awaiting response

Table 5-1 continued.

Full details of transplant regimens are given in Appendix B. MR, morphological remission (<5% bone marrow blasts); PD, progressive disease

The slow initial recruitment of patients to the study was due to the study design, which focussed solely on patients with sibling donors, demonstrating persistent morphological evidence of disease prior to HSCT and undergoing FBC-conditioned HSCT. As a result, recruitment was challenging due to the low frequency of matched sibling transplants. Furthermore, clinical practice within the department changed after the initial protocol was developed, such that patients with matched sibling donors now undergo FBATG-conditioned HSCT. This change was adopted following internal review of findings suggesting a higher risk of relapse for patients following FBC-conditioned HSCT using matched sibling as opposed to volunteer unrelated donors (VUD) (data not shown). In addition, following the publication of data from Schmid et al[311], those patients with persistent morphological disease prior to HSCT underwent FLAMSA-conditioned HSCT from 2008 onwards. Accordingly, I wrote and submitted protocol modifications that were subsequently approved by the Gene Therapy Advisory Committee, Medicines and Healthcare products Regulatory Agency and the local ethical review board that enabled recruitment to progress from 2010 onwards. These modifications included changes that substantially altered the protocol such that patients could be recruited irrespective of donor type (related or unrelated) or conditioning regimen received. Despite these efforts, as demonstrated by Table 5-1, recruitment to this study has remained challenging, primarily due to the frequent incidence of chemotherapy refractory disease at relapse post-allogeneic HSCT.

5.2.2 Characteristics, dosing and clinical course of study patients

Between January 2010 and December 2012, 4 patients were enrolled onto the study. The clinical course of each patient is outlined in Figure 5-1 and a summary of adverse events is provided in Table 5-2.

ACV and DLI as consolidation following remission re-induction

Two patients were allocated to the vaccine and DLI treatment arm: UPN 13 and UPN 22. UPN13 was a 36-year old male with a history of AML transformed from MDS (refractory anaemia with ringed sideroblasts, as per the World Health Organisation 2008 classification[1]) and poor risk karyotype (trisomies of chromosomes 8, 12 and 21) at diagnosis[312]. This patient relapsed by 6 months post-FBC RIC MUD HSCT and entered a complete morphological, but not cytogenetic, remission prior to study entry. The donor

CD3 chimerism analysis showed minimal improvement post remission re-induction therapy (donor-derived T-cells comprised 56% of CD3+ T-cells prior and 63% following FLAG chemotherapy), details of chemotherapy regimens are provided in Appendix B. This patient was assigned to the DLI and vaccine arm of the study and received a single dose of DLI ($5x10^5$ CD3+/kg) and the patient-specific vaccine ($0.5\text{-}1x10^5$ cells in 250μl). This vaccine met specifications, with CD80 expression at 47% (MFI increased 11-fold over background non-transduced cells), viability 83% by Annexin-V staining and secretion of 0.23-0.36 ng of IL-2 per 10^6 cells, per 24 hours. A total of 7 injections were administered intradermally over the patient's lower abdomen without any immediate or late adverse reactions. Unfortunately, at week 3 of the study, peripheral blood cytopenias were noted and a bone marrow biopsy confirmed relapse of AML with 24% blasts by morphology showing characteristic immunophenotypic profile. In accordance with the trial protocol, the patient was withdrawn from treatment on the study but completed follow-up assessments. The patient commenced a series of investigational agents to attempt to treat their refractory AML before ultimately succumbing to disease 11 months from the end of study follow-up and 16 months from second relapse.

Figure 5-1. Clinical course of the 4 patients who received treatment on the RFUSIN2-AML1 vaccine trial

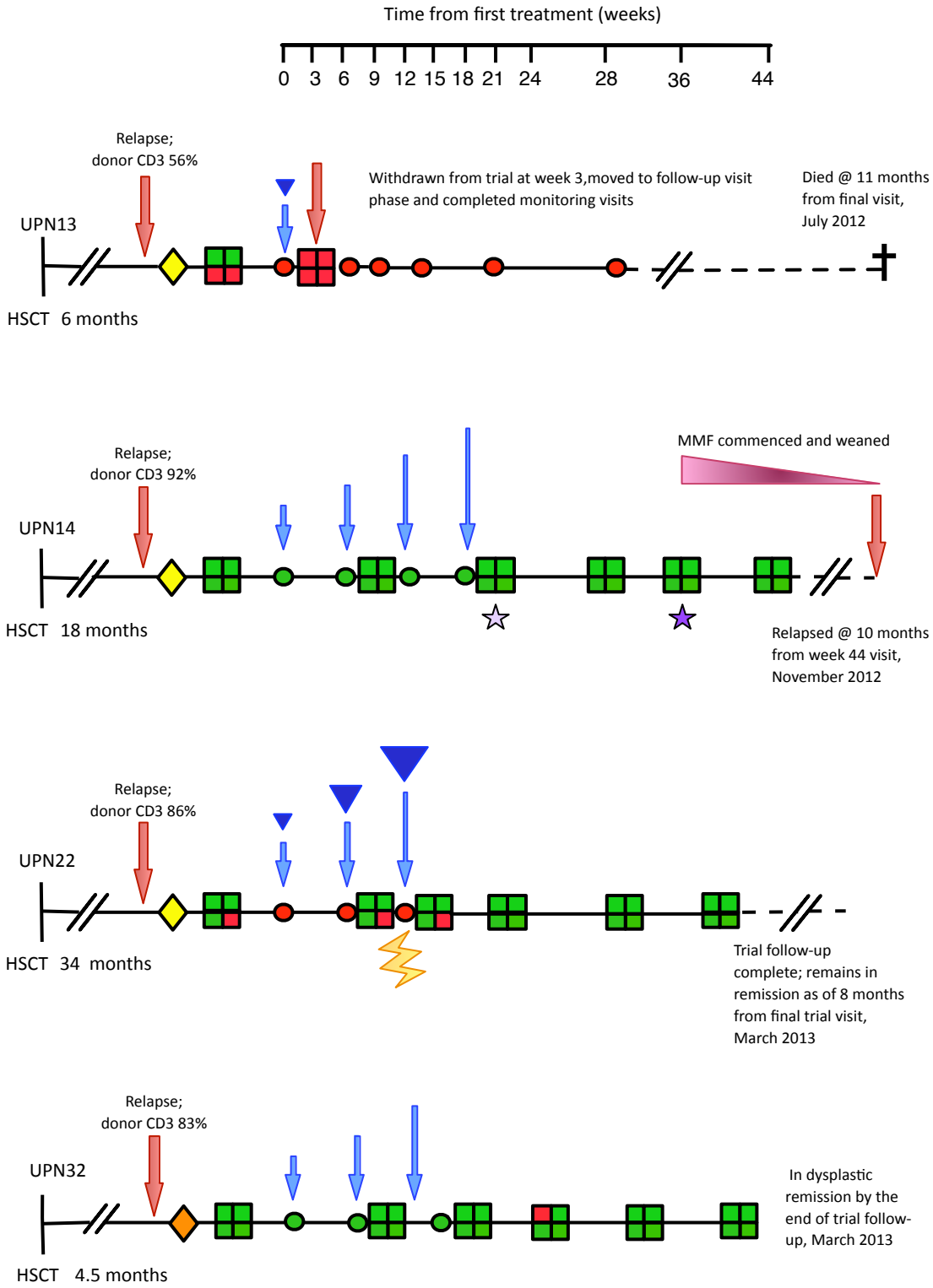
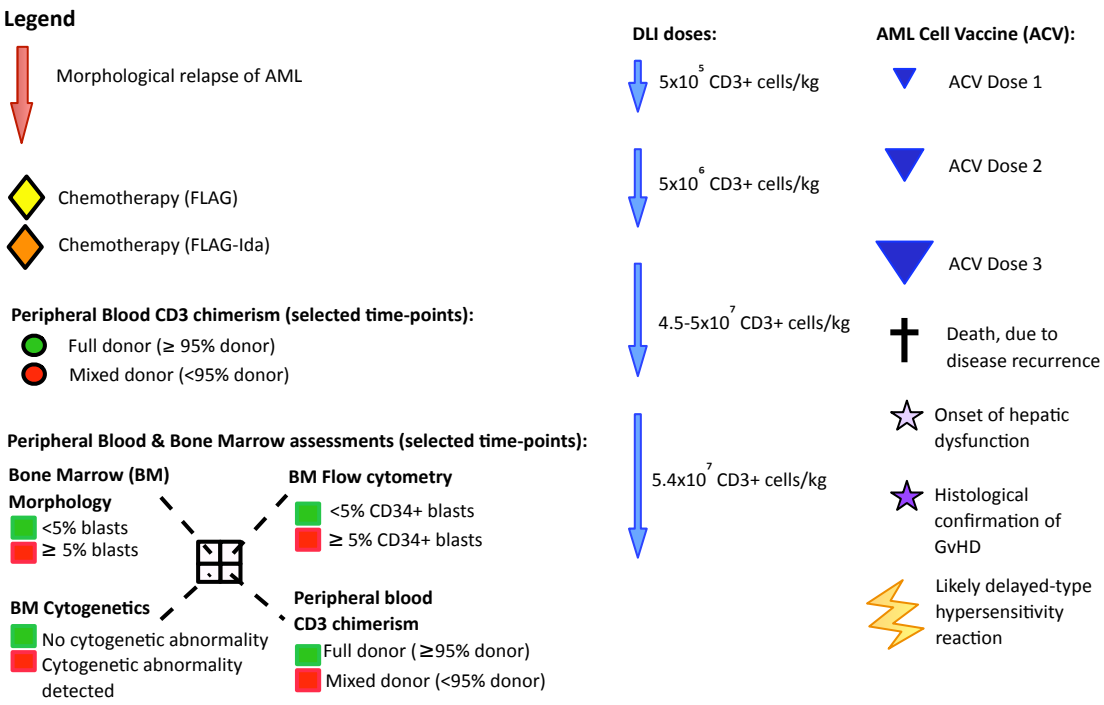


Figure 5-1 continued



UPN 22 was a 52-year old female who had AML relapse at 34 months post-FBATG HSCT from her male sibling donor for normal karyotype AML with myelodysplasia-related changes[1]. At the end of vaccine production, sufficient cell numbers for 3 doses of ACV were available. Fifty percent of the cells in the ACV product expressed CD80 (MFI increased 7-fold over control cells). The cells secreted 0.15 ng IL-2 per 10^6 cells per 24 hours and 74% of cells were viable prior to cryopreservation. Escalating doses of ACV were administered at 6-weekly intervals [0.5-1x10^5 cells (dose 1), 0.5-1x10^6 cells (dose 2) and 0.5-1x10^7 cells (dose 3)]. Each ACV administration followed same day infusion of DLI: 5x10^5 CD3+ cells/kg (dose 1), 5x10^6 CD3+ cells/kg (dose 2) and 5x10^7 CD3+ cells/kg (dose 3). The three doses of ACV were administered intradermally in the lower abdomen divided in as many sites as possible (dose 1, 14 sites; dose 2, 13 sites and dose 3, 18 sites). No treatment-related Grade 3 adverse events were recorded following administration of any of the doses of vaccine and DLI, including no fevers, chills or rigors suggestive of any systemic inflammatory response, Table 5-2. Mild induration and erythema were observed at vaccination sites 24 hours following the first and second doses of ACV, Figure 5-2 (a). Following the third dose of ACV, more pronounced local reactions were observed, which persisted approximately 96 hours post vaccination (Figure 5-2b). Similar changes were not observed in a control vaccination site injected with 0.9% normal saline, Figure 5-2 (c).

Diagnostic skin punch biopsies were collected from an un-injected site and from a vaccination site at 96 hours post dose 3. At this point, induration and erythema at vaccination sites had waned and was only just discernible. Despite this, haematoxylin and eosin stain of the punch biopsy specimen from the vaccination site demonstrated a normal epidermis and a predominantly perivascular infiltrate around both upper and lower dermal blood vessels, composed of lymphocytes with small numbers of eosinophils, mast cells and neutrophils. Immunohistochemical staining demonstrated the lymphocytic infiltrate to consist of T-cells, staining positively with CD2, CD3, CD5 and CD7 with absence of staining for B-cell (CD20, CD79a) or NK-cell (CD56) markers (data not shown). CD4+ and CD8+ T-cells were present in a 2:1 ratio. The clinical and histological findings were compatible with the development of a delayed-type hypersensitivity (DTH) reaction in UPN 22 occurring upon re-challenge with the ACV at week 12, following 2 prior vaccinations.

Figure 5-2 (a) UPN 22, week 6

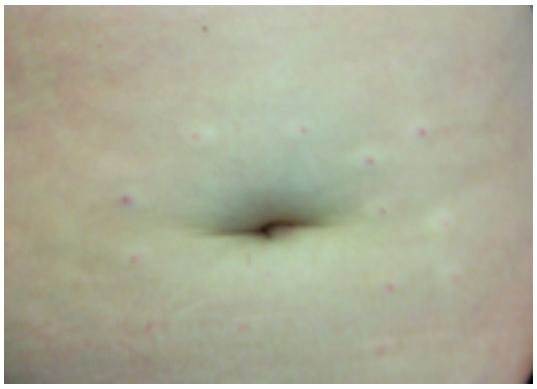


Figure 5-2 (b) UPN 22, week 12

Figure 5-2 (c) UPN 22, week 12, close-up of control and ACV sites

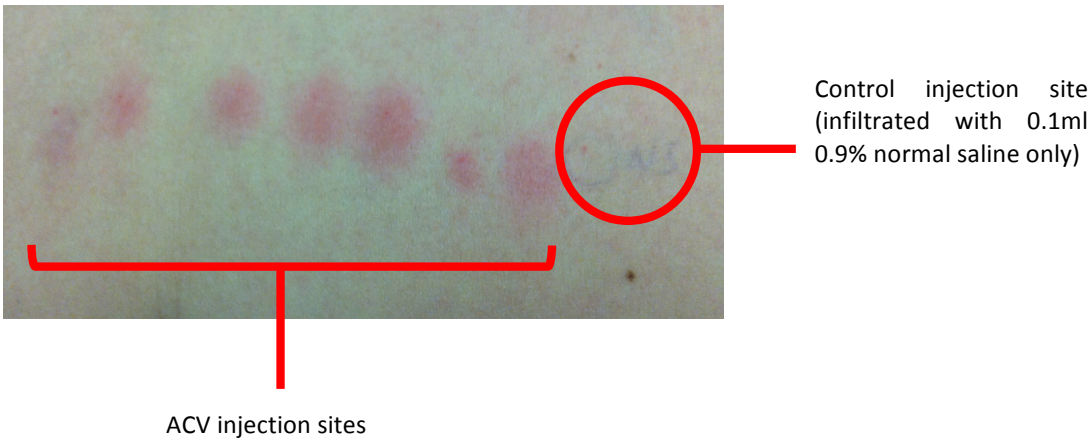


Figure 5-2 (d) Histology of skin biopsies from vaccination site, UPN 22, week 12

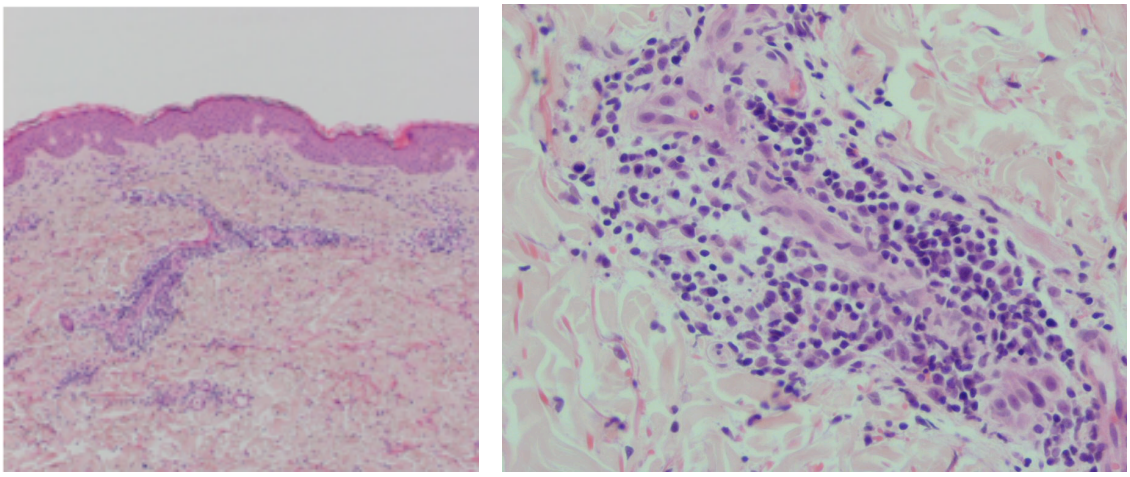


Figure 5-2 Suspected delayed-type hypersensitivity reaction in UPN 22 at 24-96 hours post third dose of ACV and DLI.
(a) Photograph of injection sites at 24 hours following ACV dose 2. (b) Photograph of injection sites at 24 hours following ACV dose 3. (c). Detail of vaccine sites, to left of umbilicus, visible in Figure 5-2 (b), demonstrating quiescent appearance of control vaccination site and erythema at ACV administration sites (d) Haematoxylin and eosin (H&E) stain of skin punch biopsy at low (left) and 20x (right) magnifications demonstrating egress of lymphocytes from a superficial vessel. H&E staining and immunostains were performed by the Department of Histopathology at King's College Hospital with morphological interpretation by Dr Sabine Pomplun.

Subsequently, UPN 22 completed all trial follow-up visits and converted to full donor chimerism (FDC) in myeloid and lymphoid lineages by week 22, which was maintained throughout the remaining follow-up period (Figure 5-1). Interestingly, Grade 2 hepatic dysfunction (by NCI CTCAE version 3 criteria, http://ctep.info.nih.gov**)** was temporarily observed at week 22 in UPN 22 (Table 5-2). This coincided with conversion to FDC. No definitive causes, such as viral hepatitis, drug reaction or anomaly on liver ultra-sound scan, were identified and no evidence of GvHD was observed. This abnormality spontaneously resolved within 3 weeks. The only other abnormality was a low-level anti-smooth muscle antibody titre (1:40) that was intermittently detected at weeks 3 and 15 that resolved by week 18 and remained undetectable thereafter. There was no clinical evidence of autoimmune toxicity. Grade 1 neutropenia was detected between weeks 6 and 22, resolving by week 30. Bone marrow cellularity was maintained at 40-50% at all time points with no evidence of bone marrow failure. The patient was free from GvHD and in morphological remission with full donor chimerism at the time of writing, 18 months following AML relapse.

DLI without vaccine as consolidation following remission re-induction

UPN 14 was a 44-year old female with a history of de novo, intermediate cytogenetic risk[2], AML with maturation[1], transplanted in CR2 using a sibling donor. Following the documentation of mixed chimerism, this patient received 2 doses of pre-emptive DLI at 7 and 10 months post-HSCT. Despite an improvement in donor CD3% from 48% to 83% at 3 months following the second DLI dose, UPN14 relapsed morphologically at 18 months post-HSCT. Following assignment to the DLI arm of the vaccine trial, the patient received a total of 4 escalating doses of DLI. Due to lack of availability of a dose at $1x10^8$ CD3+ cells/kg, the patient received $5.4x10^7$ CD3+ cells/kg as the fourth dose at week 18. At 3 weeks following the final dose of DLI, UPN 14 developed hepatic dysfunction (maximum severity Grade 3 by NCI CTCAE version 3 criteria). This progressed and the patient additionally experienced other features compatible with a diagnosis of chronic GvHD, including mild sicca syndrome and oral ulceration. A liver biopsy demonstrated peri-portal inflammation, most consistent with GvHD. This development was classed as the first dose limiting toxicity (DLT) of the study, in accordance with the trial protocol. Additionally, anti-nuclear antibodies (nucleolar pattern, titre 1/160) were observed in this patient at week 36 but were not detected at week 44. The patient was treated with single-agent immunosuppressive therapy, with good

response, but unfortunately experienced extramedullary (gingival) relapse of AML, 2 years after the initial relapse, and remains on active treatment at this time.

The fourth patient, UPN 32, a 55-year old male, experienced early recurrence of AML with myelodysplasia related changes[1] 6 months following FB4ATG-conditioned HSCT from their male sibling donor. Three doses of DLI alone were well tolerated by this patient. Due to absence of availability of $5x10^7$ CD3+ cells/kg, the final dose of DLI in UPN 32 was $4.5x10^7$ CD3+ cells/kg. UPN 32 experienced no treatment-related Grade 3 adverse events although asymptomatic Grade 1-2 neutropenia was detected between weeks 6-25 of treatment and was most likely reflective of persistent dysplasia within the bone marrow. A bone marrow biopsy at week 25 suggested a morphological excess of blasts (7%) that was not corroborated by other diagnostic modalities (cytogenetic, immunophenotyping and chimerism studies) and on subsequent repeat bone marrow studies, no excess of blasts was confirmed. Incidental detection of anti-nuclear antibodies (nucleolar staining pattern, titre ranging from 1/80 to 1/1280) was observed in UPN 32 from week 9 onwards and persisted until end of follow-up. By March 2013, the patient completed monitoring, remaining in dysplastic remission from their disease and exhibited FDC in myeloid and lymphoid lineages.

Table 5-2 Adverse events recorded for patients treated on the RFUSIN2-AML1 trial according to NCRI CTCAE version 3

Adverse event and maximum grade experienced (where applicable)	DLI only (n=2) arm Total number of events	DLI and vaccine (n=2) arm Total number of events
Haematological toxicity: Grade 1-2 Grade 3-4	 1 0	 1 1¶
Infection: Colitis with Grade 1-2 neutrophils URTI (presumed viral), Grade 2 Cellulitis, Grade 2 Intravenous catheter-related infection, Grade 3	 1¥ 1 0 0	 0 0 1 1
Asymptomatic autoimmune reaction: Grade 1 Nature of the antibodies: Anti-smooth muscle antibody Anti-nuclear antibody	2 0 2**	1 1* 0
Hepatic dysfunction: Grade 1-2 Grade 3-4	 0 1	 1 0
Acute graft-versus-host disease: Chronic graft-versus-host disease: (liver, oral, genital tract)	0 1	0 0
Dyspnoea (during DLI): Grade 1-2 Hypoxia (during DLI): Grade 1-2	 1 0	 1 1
Skin rash or lesion (including vaccine site bruising and/or induration): Grade 1-2 Grade 3-4	 1 0	 2 1§
Nausea and/or vomiting: Grade 1-2 Constipation: Grade 1-2 Diarrhoea: Grade 1-2	 0 0 2¥	 2¶ 2¶ 0
Joint and/or muscle aches/pains: Grade 1-2	 1	 1
Fatigue: Grade 1-2	 1	 2

CTCAE, Common Terminology Criteria for Adverse Events; DLI, Donor Lymphocyte Infusion; URTI, Upper Respiratory Infection

¶ Adverse events were recorded during the follow-up phase for UPN13, during which period the patient had active disease and was undergoing a range of drug treatments responsible for the majority of the documented adverse events. Apart from Grade 1 thrombocytopenia, all haematological toxicity was experienced following withdrawal from treatment on trial

§ UPN22 had developed a basal cell carcinoma during remission re-induction therapy that was excised during the course of the trial

¥ UPN32 had microbiologically confirmed colitis (due to *Salmonella ridge)* that was treated with appropriate antibiotics

* Anti-smooth muscle antibody in UPN22, highest titre 1/40

**Anti-nuclear antibody (ANA) in UPN14, highest titre 1/160 with nucleolar pattern; ANA in UPN32, highest titre 1/1280 with nucleolar pattern.

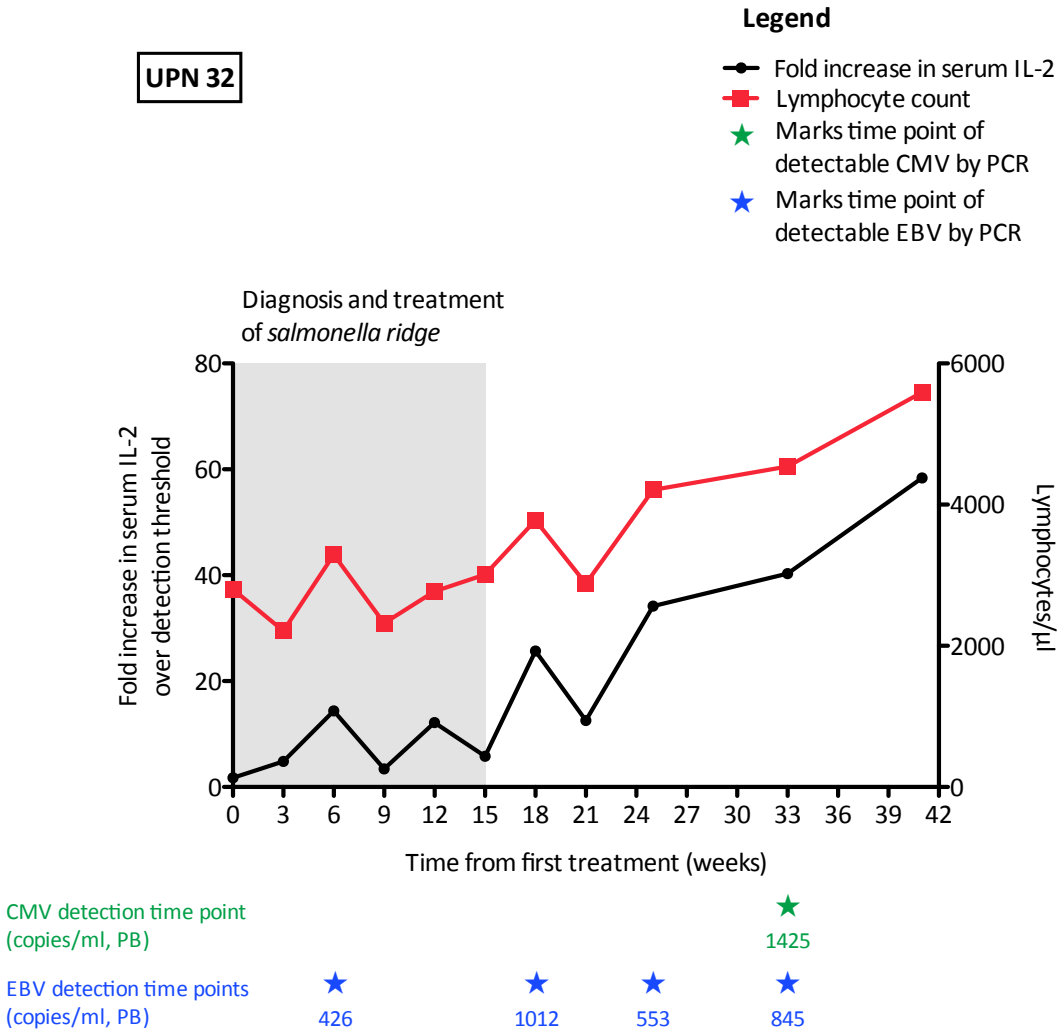
5.2.3 Absence of replication competent lentivirus generation or systemic elevation of serum IL-2 in vaccine-treated patients suggests safety of the AML Cell Vaccine

In addition to clinical safety monitoring, samples were collected at defined time-points to exclude the generation of replication competent lentivirus (RCL) or systemic elevation of serum IL-2 levels following administration of ACV. No patients in either arm demonstrated evidence of RCL, as demonstrated by (1) absence of viral integration into peripheral blood or bone marrow mononuclear cells (using virus-specific qPCR analysis of genomic DNA from these compartments); (2) absence of viral RNA in patients' sera (using virus-specific RT-qPCR studies) and (3) absence of detectable HIV p24 antigen in patients' sera by ELISA studies (see Appendix D for data). Drs YuQian Ma and Sabine Domning performed and analysed the safety studies described above. UPN 32 (who was not allocated to the vaccine treatment arm) demonstrated marginally elevated levels of HIV p24 antigen at weeks 6 and 33 that was not corroborated by analyses of HIV 1&2 p24 antigen performed at the South London Specialist Virology Centre laboratories at King's College Hospital, London. The result was therefore likely due to high background signal associated with this particular assay.

Serum IL-2 levels were assessed by ELISA to exclude the possibility of systemic elevation of IL-2 due to secretion by ACV. UPNs 13, 14 and 22 demonstrated no elevation of serum IL-2 above the threshold for detection by ELISA at each consecutive time point. UPN 32 had consistently elevated serum IL-2 levels during the course of the trial (Figure 5-3). Since the patient did not receive ACV, the IL-2 must be derived from immune cells, such as T- and NK-cells, possibly in response to infection. Indeed, Figure 5-3 shows a parallel rise in lymphocyte numbers and serum IL-2 levels, which supports the possibility of rising IL-2 levels due to increased circulating immune cells. Results to be presented later showed that the rise in lymphocyte numbers was due to increased numbers of terminal effector CD8+ T-cells (Figure 5-8, section 5.2.4).

Infectious organisms identified in this patient included *salmonella ridge*, isolated from diarrhoeal stool cultures from the patient prior to study entry. Since the enteric symptoms responded completely to antibiotic therapy within the first 15 weeks of the trial period, they are unlikely to account for the subsequent rise in lymphocyte numbers and IL-2 levels. This patient also experienced fluctuating low-level reactivation of viruses including EBV and CMV (determined by PCR performed at the South London Specialist Virology Centre laboratories at King's College Hospital, London) during the course of follow-up. Both UPN 32 and their sibling donor had previously been exposed to EBV and CMV, demonstrable by the detection of EBV and CMV specific IgG antibodies in their sera. UPN 32 was therefore at high risk of experiencing reactivation of these viruses, due to transplant-related immunosuppression and lymphodepletion, and had already experienced CMV reactivation requiring antiviral drugs prior to study entry. During trial follow-up, CMV was detectable only at week 33 (1425 copies/ml) and low-level EBV viraemia was observed predominantly from week 18 onwards (Figure 5-3). The substantial population of terminal effector CD8+ T-cells present within UPN 32 at study entry (Figure 5-8) and the controlled, low-level viraemia observed during follow-up suggest that effective anti-viral T-cell immunity may have been transferred with the infused product at the time of HSCT.

Figure 5-3 Fold increase in serum IL-2 detected in UPN 32 at trial monitoring time points and parallel rise in lymphocyte numbers.



Serum IL-2 concentration (pg/ml) was measured using ELISA. Results are normalised to the threshold of detection, which showed inter-assay variation (ranging from 4.87 pg/ml to 36.5 pg/ml, see Appendix D). The grey panel outlines the time course of *salmonella ridge* infection. Time points where EBV and CMV reactivation were detected by PCR studies during monitoring are also indicated (blue and green stars respectively) along with the copy numbers of each virus detected per ml in the peripheral blood (PB).

5.2.4 Analysis of lymphocyte subset composition in trial subjects following ACV and/or DLI treatment

Prolonged deficiencies in immune subset composition even late post-HSCT have been reported[313,314], including in the naïve T-cell compartment which is particularly important for promotion of GvL[42,300,301]. One approach, considered during the design of this trial, to potentially overcome this issue, has been to infuse donor lymphocytes alongside vaccine administration. However, to specifically address whether lymphocyte subset deficiencies were present in subjects during therapy, their lymphocyte composition prior to and during the course of treatment were assessed. Comparison was made with results from 11 healthy, age-matched volunteers. Measurement of frequencies and numbers of lymphocyte subsets and expression of activation markers was undertaken (Tables 2-10 and 2-11, Chapter 2). Study of T- and NK-cell populations was of particular interest given the prior *in vitro* data demonstrating the ability of CD80/IL-2 modified AML blasts to activate leukaemia-specific cytotoxicity by these cell types[120,121]. Lymphocyte subset analysis for UPN 13 is not discussed here. This is because the rapid relapse following just one dose of DLI and vaccine would confound evaluation of the impact of ACV and DLI, due to the immunosuppressive nature of salvage chemotherapy and active leukaemia.

5.2.4.1 A rise in lymphocyte numbers following trial therapy is observed in UPNs 22 and 32

Sequential peripheral blood lymphocyte numbers post-HSCT were available for UPNs 14, 22 and 32 during routine follow-up after stem cell infusion, at relapse and over the trial monitoring period (Figure 5-4 a and b). Lymphocyte numbers in UPN 22 recovered to within the normal range observed in the 11, healthy, age-matched volunteers, from around 1 year post-HSCT (Figure 5-4a). The decline in absolute lymphocyte count after relapse was likely due to the lymphodepleting effects of salvage chemotherapy. UPN 22's lymphocyte count rose during the course of ACV and DLI administration but remained below the range for healthy controls at the end of the trial monitoring period.

The lymphocyte numbers in UPN 14 showed a gradual recovery following HSCT almost attaining the range seen in the healthy controls. Following 2 doses of DLI for mixed chimerism, a small rise in the lymphocyte numbers was noted. These numbers remained unchanged after relapse and receipt of DLI on trial, despite onset of cGvHD and commencement of immunosuppressive therapy. Lymphocyte numbers below the

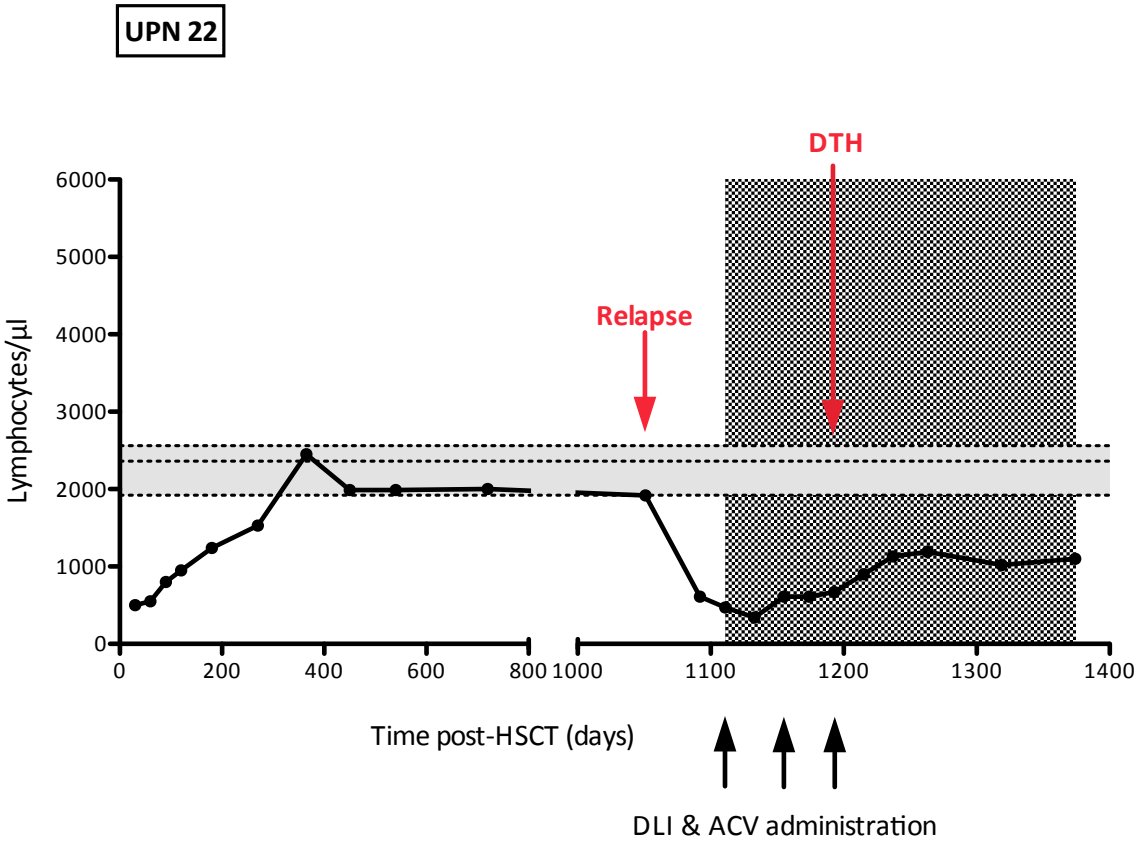
range for healthy age-matched volunteers were observed in UPN 32 during the early post-HSCT period (Figure 5-4b). Following salvage chemotherapy and during the course of the trial, a marked rise in lymphocyte numbers was observed. This may have been due to ongoing lymphocyte reconstitution post-HSCT, increase in lymphocyte numbers following infusion of new donor lymphocytes or expansion of CD8 T cells due to antigenic stimulation, for example by the herpes viruses present in this patient (described in 5.2.3).

The lowest pre-study baseline lymphocyte count was observed in UPN 22 (absolute lymphocyte count 470 lymphocytes/μl) whilst baseline lymphocyte counts were 1520 lymphocytes/μl and 2800 lymphocytes/μl in UPNs 14 and 32 respectively. Lymphocyte numbers in UPN 14 remained unchanged from the pre-study levels for the duration of follow-up. Two of the 3 patients, UPN 22 and UPN 32 demonstrated a doubling in their absolute lymphocyte numbers by the end of the follow-up period relative to the start of study (1100 lymphocytes/μl in UPN 22 and 5590 lymphocytes/μl in UPN 32). However, the rise in lymphocyte numbers seen in UPN 22 only partially corrected the deficiency in the count observed following salvage chemotherapy for relapse.

To summarise, all 3 patients demonstrated lymphopenia early post-HSCT. A rise in lymphocyte numbers during the course of therapy on trial was observed for 2 of the 3 patients, of whom one experienced a rise in lymphocyte numbers to well above the range seen in healthy age-matched volunteers.

Figure 5-4 (a & b). Absolute lymphocyte counts in subjects during post-transplant recovery, at relapse and in the course of trial monitoring.

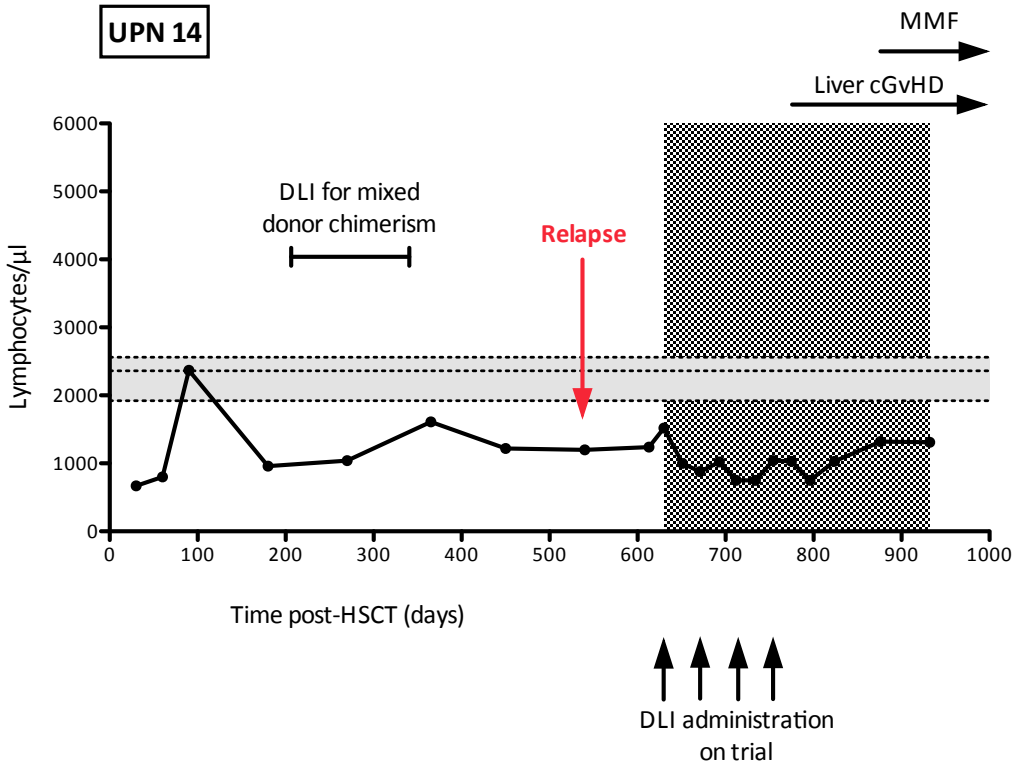
a) Sequential lymphocyte counts in UPN 22 (recipient of DLI and the AML Cell Vaccine) post-HSCT, upon relapse and during trial monitoring

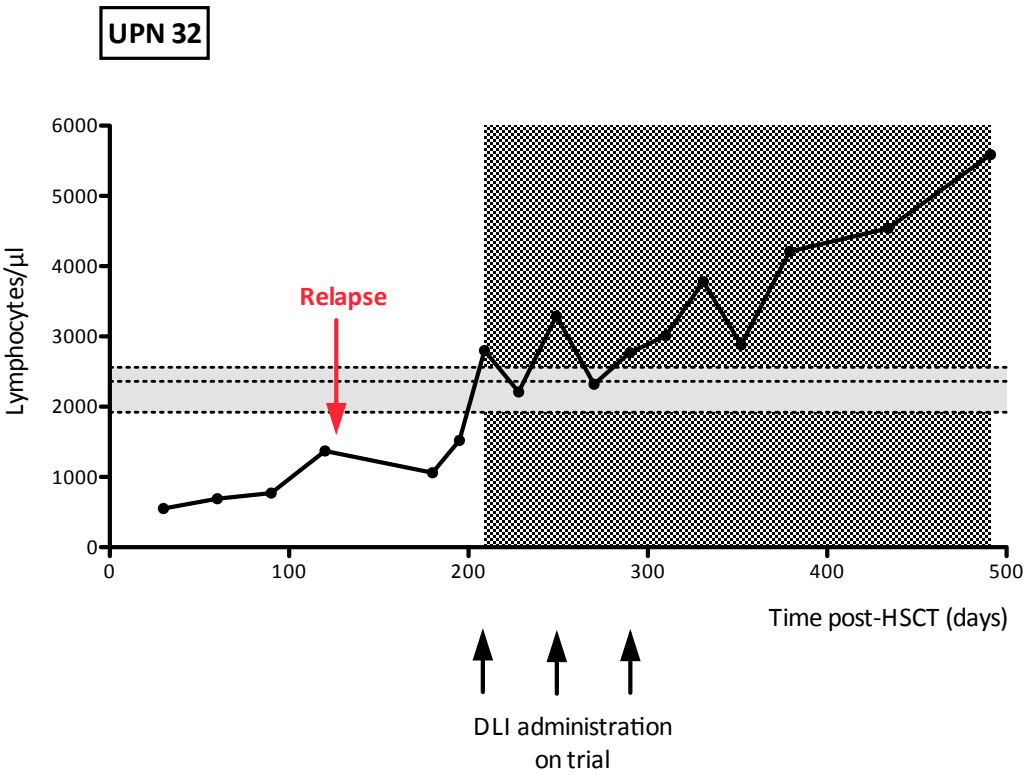


Horizontal dotted lines encompassing light grey shaded areas mark median and interquartile range of lymphocyte counts in eleven age-matched healthy volunteers. The cross-hatched area highlights the period of treatment and follow-up on the RFUSIN2-AML1 vaccine trial. Upwards arrows denote administration of DLI with/without ACV according to the protocol and treatment assignment. Results for UPN 22 (recipient of DLI and ACV, 5-4a) are shown on this page and overleaf for UPNs 14 and 32 (who received DLI only, 5-4b).

DTH, delayed type hypersensitivity reaction; DLI, donor lymphocyte infusion; ACV, AML Cell Vaccine; MMF, mycophenolate mofetil; cGvHD, chronic Graft-versus-Host Disease.

(b) Sequential lymphocyte counts in UPNs 14 and 32 (recipients of DLI only) post-HSCT, upon relapse and during trial monitoring





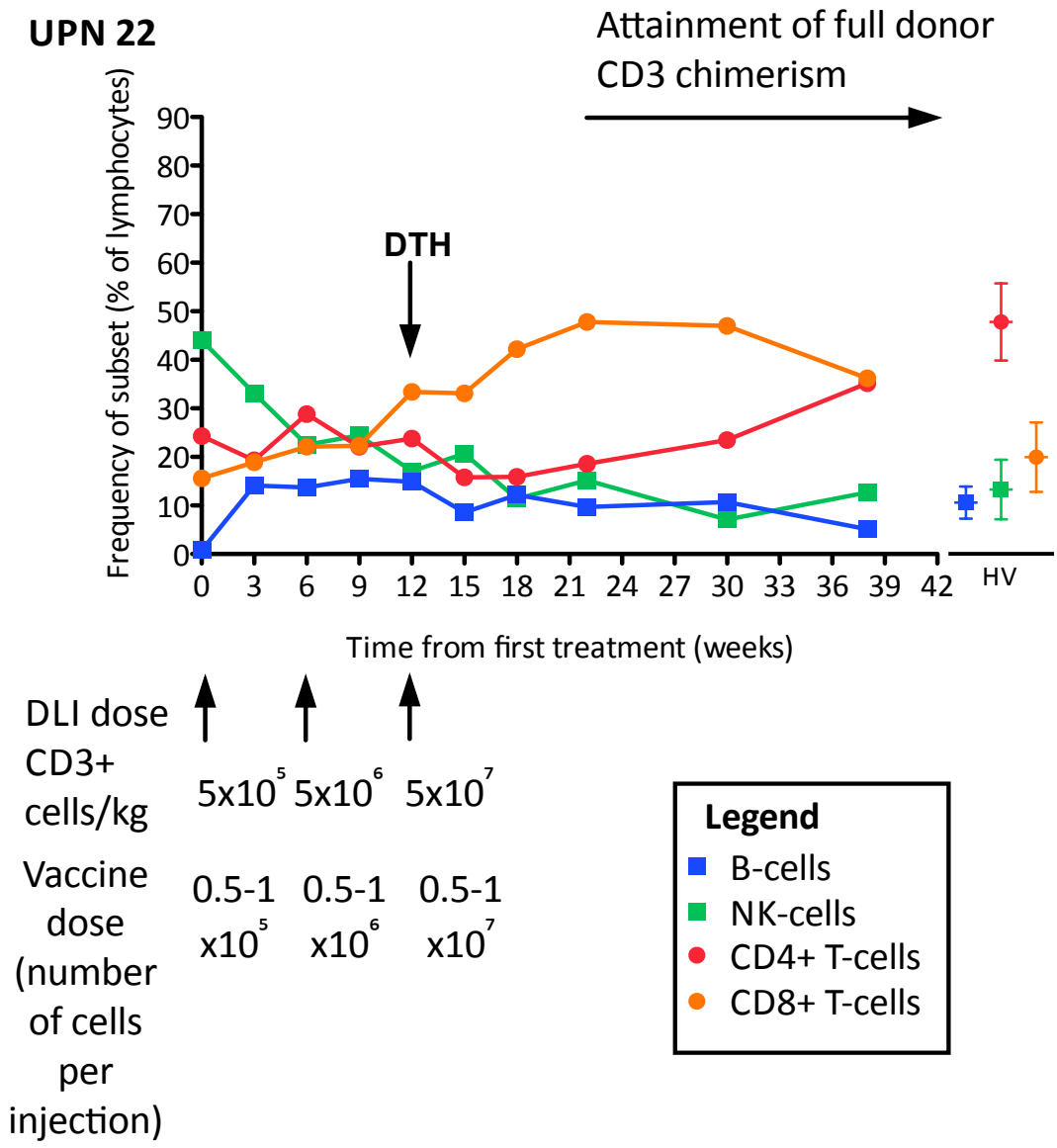
5.2.4.2 Lymphocyte subset composition in trial subjects differs from healthy volunteers and remains abnormal during therapy

More detailed analyses of the frequencies and numbers of individual lymphocyte subsets were performed, and are shown for each subject in Figures 5-5 and 5-6. Lymphocyte subset composition in the trial subjects differed from that in healthy volunteers, with the greatest divergence observed in the numbers and frequencies of the CD4+ T-cell population. CD4+ T-cells accounted for the major lymphocyte subset in healthy volunteers (mean 48% of all lymphocytes, Figure 5-5). In UPNs 14, 22 and 32 the CD4 frequencies were 11%, 24% and 12% respectively prior to trial therapy. At the end of the trial monitoring period, the CD4+ T-cell percentages had increased in UPNs 14 and 22 but remained lower than the mean observed in healthy volunteers, accounting for 21% of all lymphocytes in UPN 14, 35% in UPN 22 and 3% in UPN 32. This deficiency in the CD4+ T-cell populations reversed the normal CD4:CD8 ratios. The mean ratios during follow-up were 0.64 (range 0.28-1.0) for UPN 14, 0.83 (range 0.38-1.56) for UPN 22 and 0.1 (range 0.05-0.18) for UPN 32, contrasting with the mean CD4:CD8 ratio of the 11 healthy age-matched volunteers of 2.83 (range 0.83-5.70). A lag in recovery of CD4+ T-cell numbers behind that of other lymphocyte subsets following allogeneic transplantation[315-317], resulting in an inverted CD4:CD8 ratio[313,318,319], has been previously reported by other groups.

The numbers of B- and NK- cells and CD8+ T-cells in UPN 14 were similar to those in healthy volunteers throughout the trial monitoring period (Figure 5-6). Therefore, the low lymphocyte numbers in this patient prior to and during receipt of DLI are attributable to the deficiency in the CD4+ T-cell population. Numbers of B- and NK- cells in UPN 22 were below that observed in healthy volunteers throughout the trial follow-up period, whilst numbers of CD8+ T-cells were near the range seen in the healthy volunteers. The high lymphocyte count observed throughout therapy with DLI in UPN 32 was due to a large expansion of CD8+ T-cells that was evident prior to DLI administration and persisted throughout follow-up. The mean CD8+ T-cell percentage amongst lymphocytes in the healthy volunteers was 19.8%. At the time of trial entry, the proportion of CD8+ T-cells amongst the lymphocyte population in UPN 32 was 73.5% and consistently comprised 70-80% of all lymphocytes before falling somewhat to 60% by the end of follow-up (Figure 5-5). This patient also showed a rise in B-cell numbers and frequency to just above the range seen in the healthy volunteers during the follow-up period after completion of DLI. In summary, lymphocyte

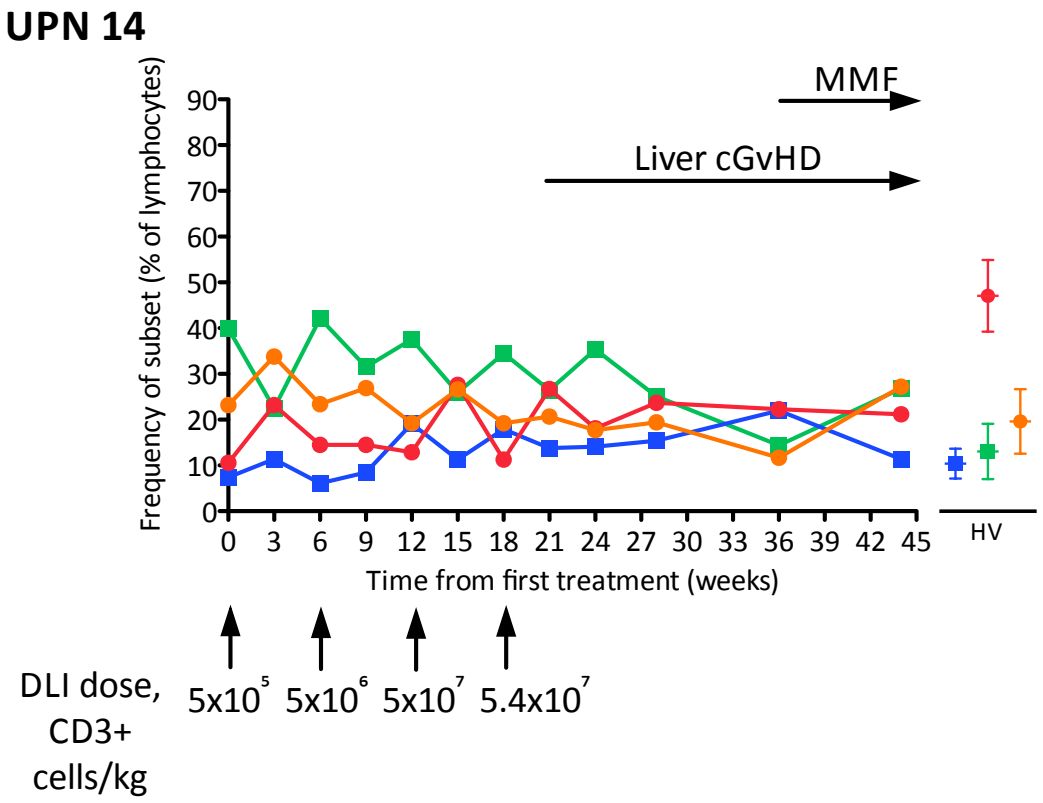
subset composition was abnormal in all subjects during the course of therapy and follow-up and was not altered substantially following administration of ACV and/or DLI (with the exception of the increase in B-cells and CD8+ T-cells in UPN 32).

Figure 5-5 Lymphocyte subset composition in UPNs 14 & 22 is similar to that in healthy volunteers



Lymphocyte subset frequencies were sequentially analysed in trial subjects during the course of treatment and follow-up. Results are shown for the single recipient of DLI and ACV (UPN 22) and the two recipients of DLI only (UPNs 14 &32) on this and the following page respectively. Mean and standard deviation are shown in each plot for 11, healthy, age-matched controls.

Figure 5-5 continued.



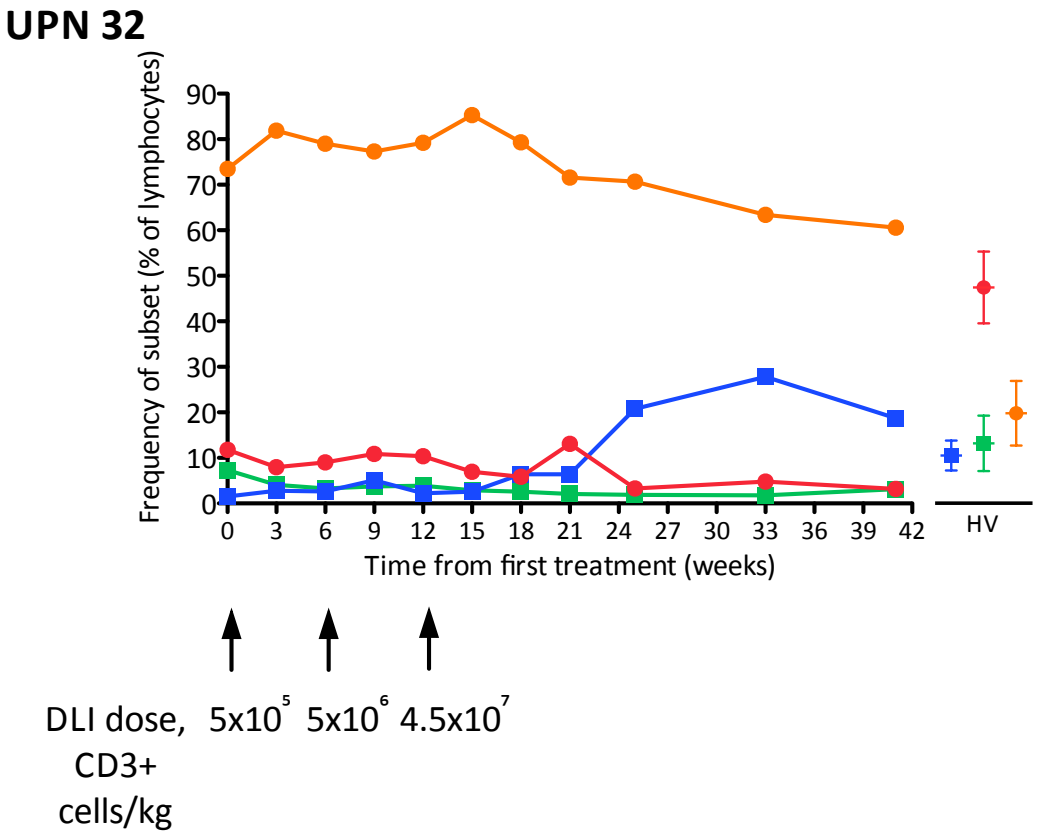
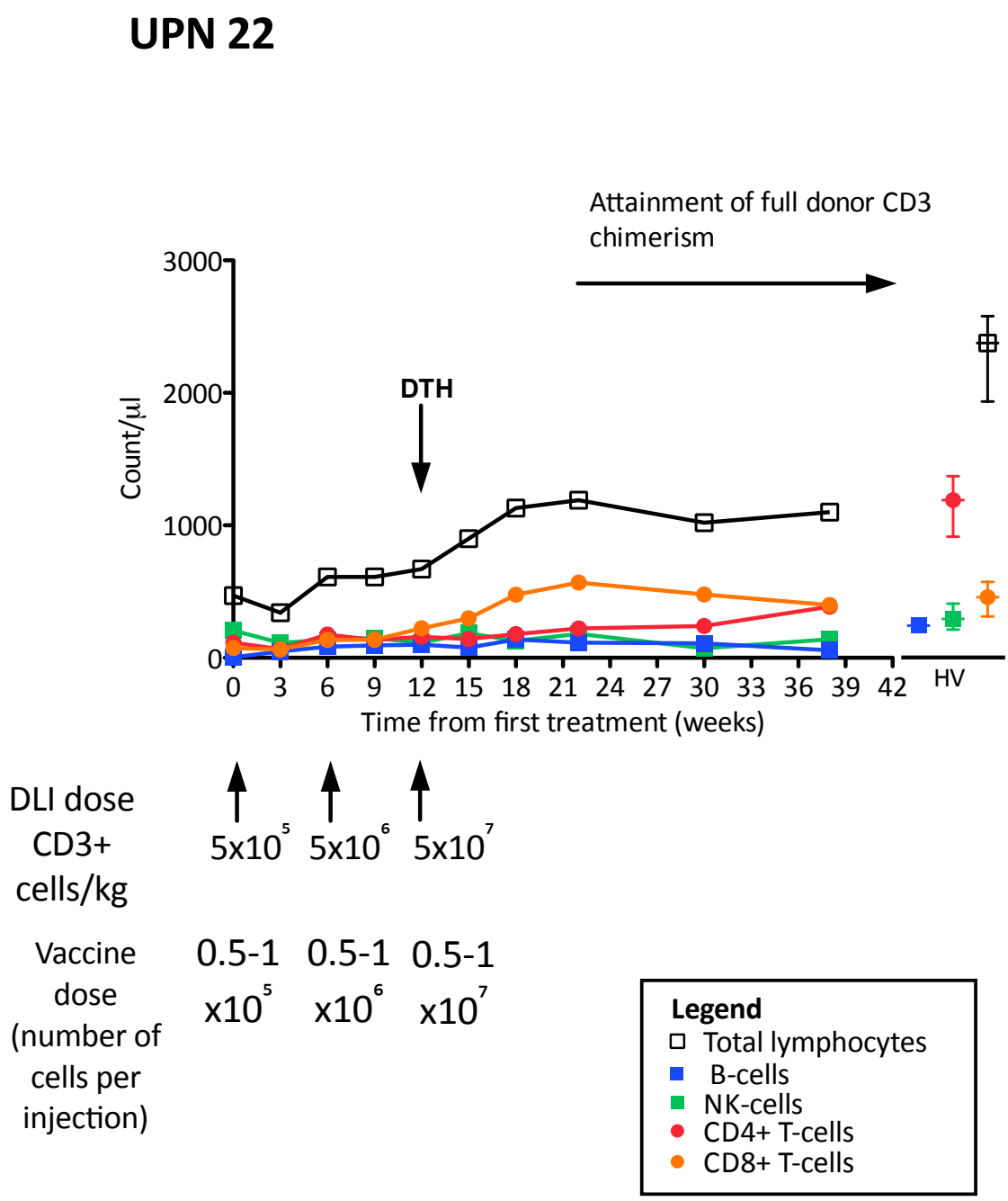
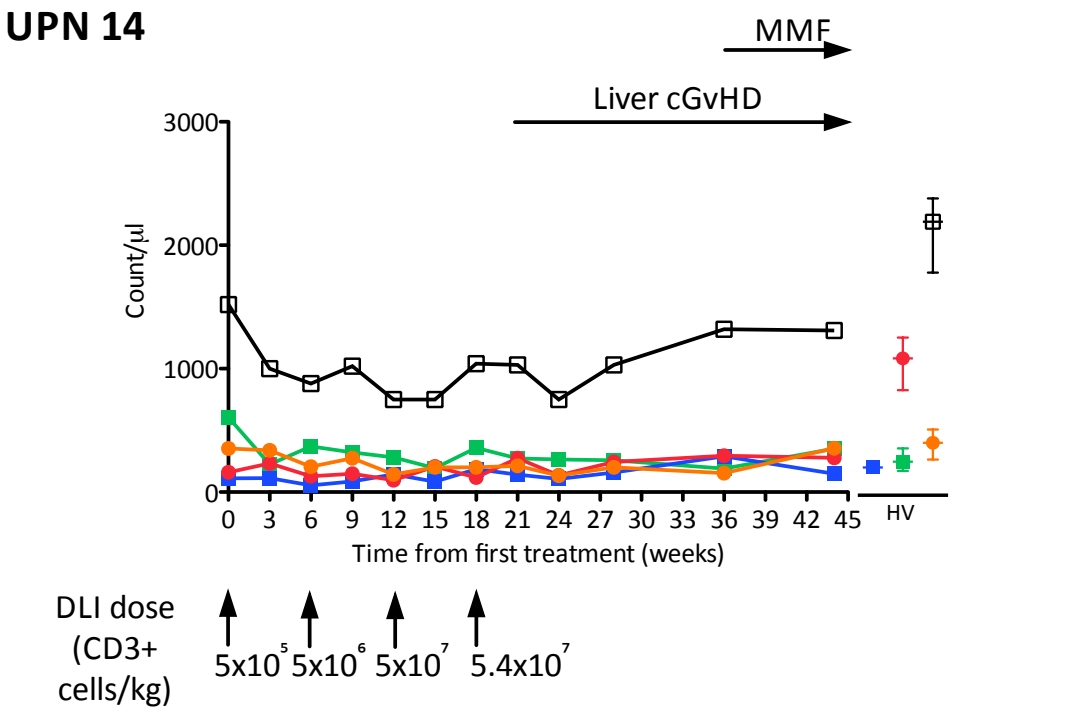


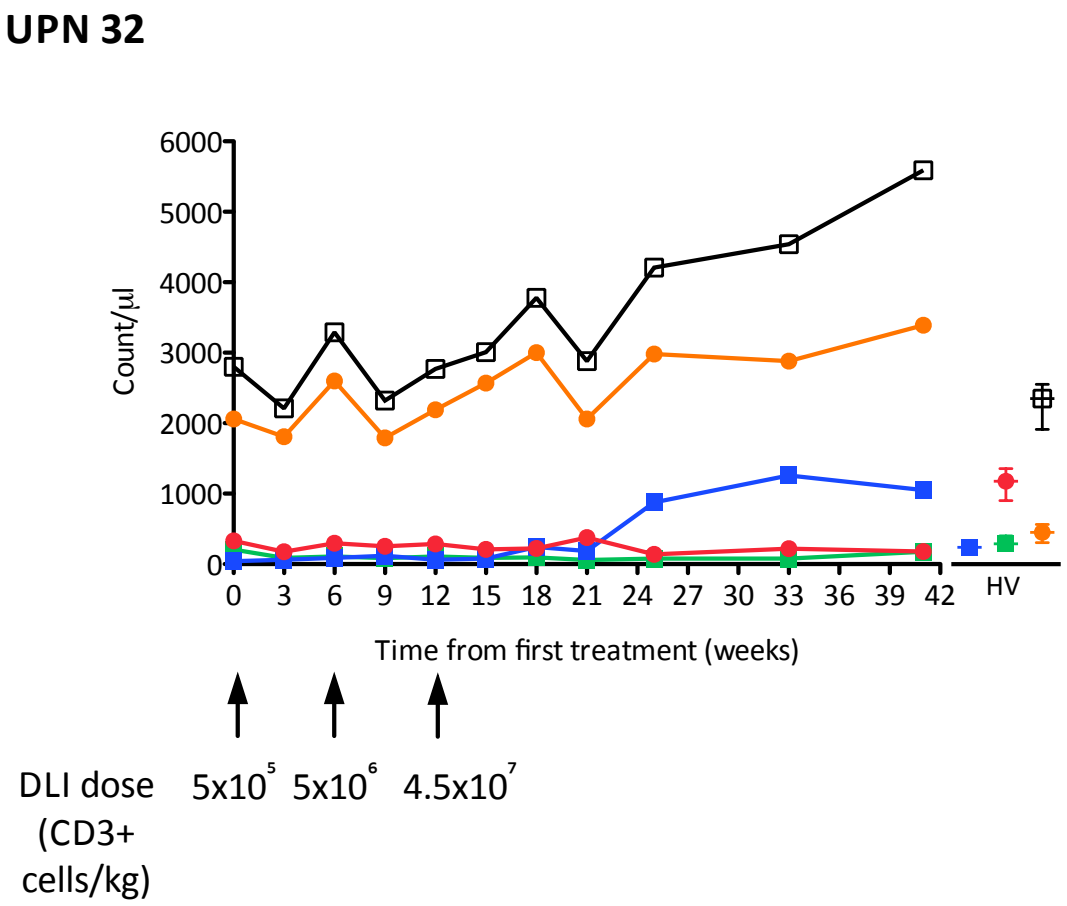
Figure 5-6 Sequential analysis of lymphocyte subset numbers reveals reduced CD4+ T-cell numbers in all trial subjects relative to healthy volunteers.



Absolute numbers of lymphocyte subsets were monitored sequentially in trial subjects during the course of treatment and follow-up. Results for the recipient of DLI and ACV (UPN22) and the two recipients of DLI only (UPNs 14 and 32) are shown on this page, and the following page, respectively. Mean and standard deviation are shown in each plot for 11, healthy, age-matched volunteers (HV).

Figure 5-6 continued

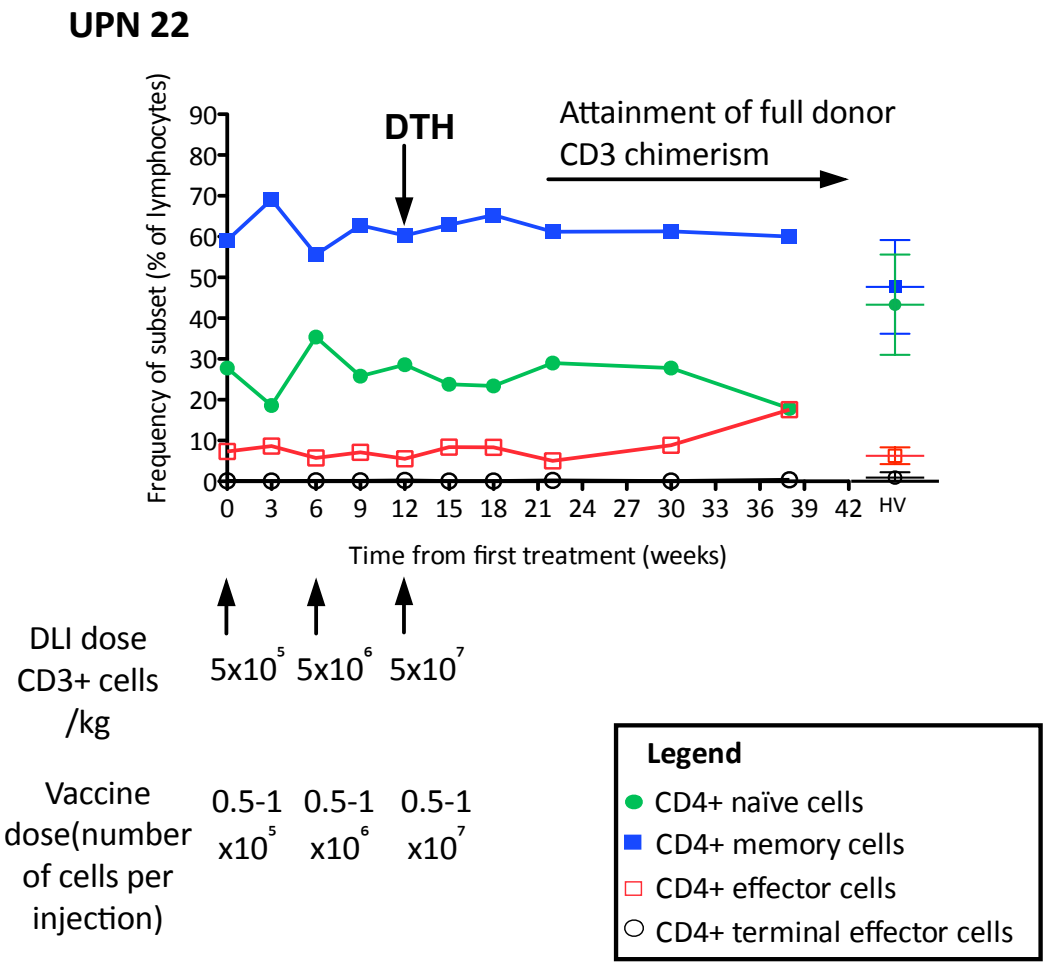




5.2.4.3 CD4+ and CD8+ T-cell subset composition is unchanged in subject during trial therapy

CD4+ and CD8+ T-cell subset compositions were also assessed (Figures 5-7 and 5-8 respectively). Overall, individual subsets showed stable frequencies over the course of follow-up in all 3 patients. CD4+ and CD8+ naïve, memory and effector T-cell subset frequencies were most similar to the healthy volunteers in UPNs 14 and 22. Both of these patients were at least 1 year post allogeneic HSCT, possibly explaining the near normal frequencies in these patients. By contrast, UPN 32 (who had undergone allogeneic HSCT less than 1 year prior) differed most profoundly from healthy volunteers, with very high frequencies of CD4+ effector and CD8+ terminal effector T-cells and low frequencies of CD4+ or CD8+ naïve T-cells. Analysis of HLA-DR expression on CD4+ and CD8+ T-cells in UPN 32 was used to assess activation status of these subsets[320]. High expression of HLA-DR on over 80% of CD4+ and CD8+ T-cells was observed at study baseline in this patient and waned during the course of follow-up (Figure 5-9). By contrast, the mean percentages of DR+ T-cells during follow-up on trial were 24% (CD4+) and 38% (CD8+) in UPN 14 and 30% (CD4+) and 57% (CD8+) in UPN 22, with little change during the course of therapy (data not shown). The activated profile and skewing of the T-cell subsets again identifies activation and expansion of effector T-cells in UPN 32. This could reflect ongoing activation of effector cells as a result of recent transplant conditioning and chemotherapy or potentially responses against antigenic, microbial stimuli e.g. herpes viruses, *salmonella* spp, as described earlier (section 5.2.3)

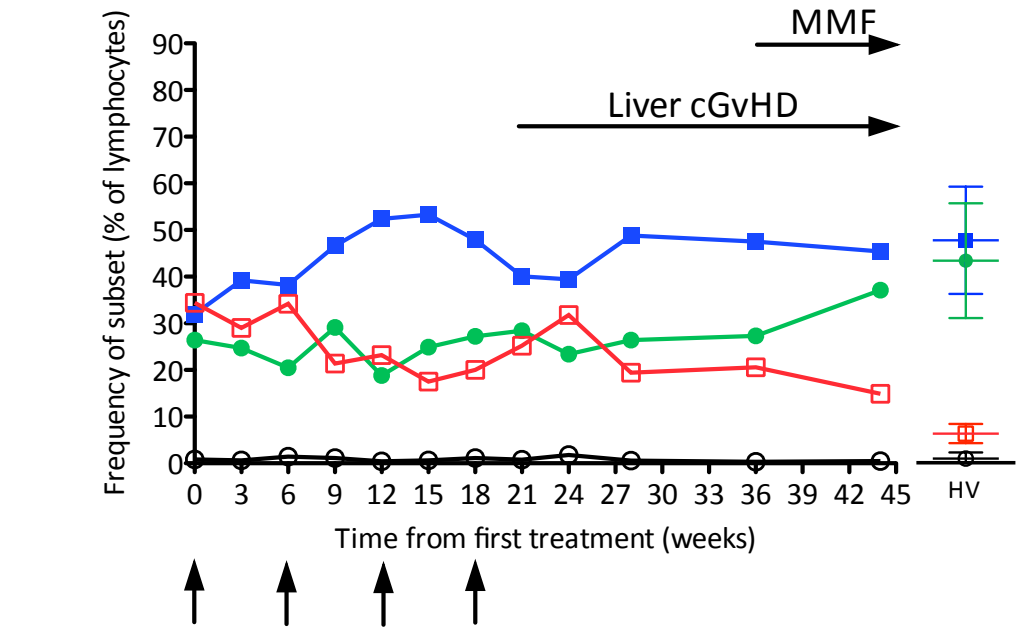
Figure 5-7 Despite a numerical reduction in CD4+ T-cells, CD4+ T-cell subset composition is similar to controls in UPNs 14 & 22.



CD4+ T-cell subset composition was determined for each trial subject during treatment and follow-up. Results for the recipient of DLI and ACV, UPN 22, are shown on this page and for the two recipients of DLI alone (UPNs 14 and 32) on the next page. Mean and standard deviation of CD4+ T-cell subset frequencies in 11 age-matched volunteers is shown in each plot.

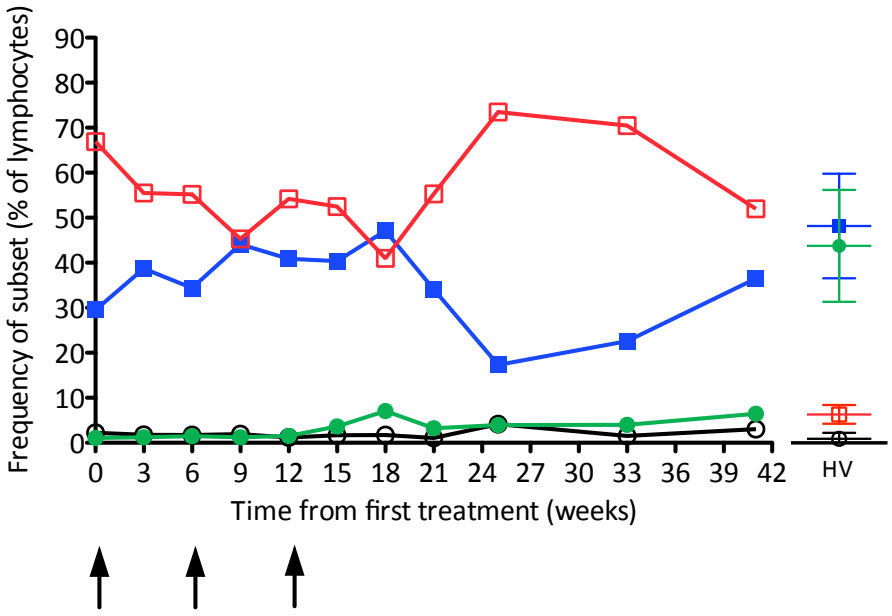
Figure 5-7 continued

UPN 14



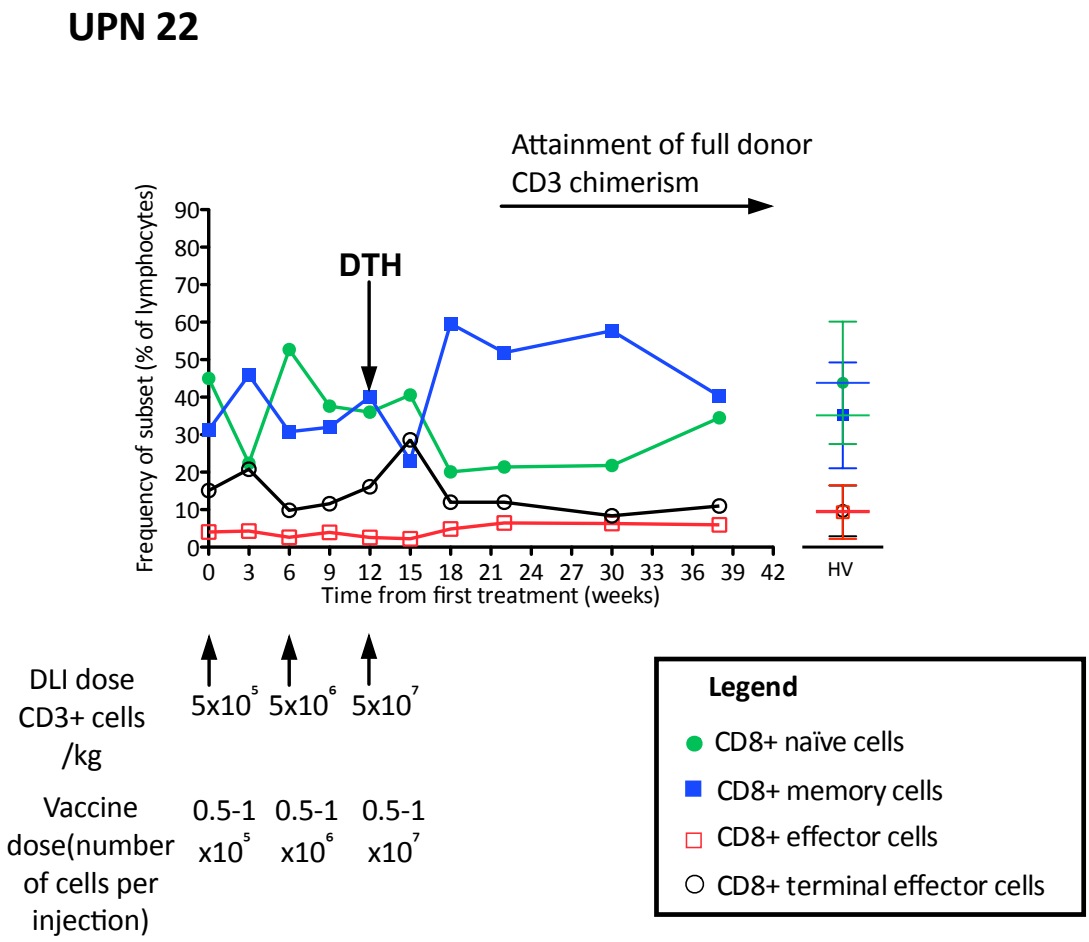
DLI dose, CD3+ cells/kg: $5x10^5$ $5x10^6$ $5x10^7$ $5.4x10^7$

UPN 32



DLI dose, CD3+ cells/kg: $5x10^5$ $5x10^6$ $4.5x10^7$

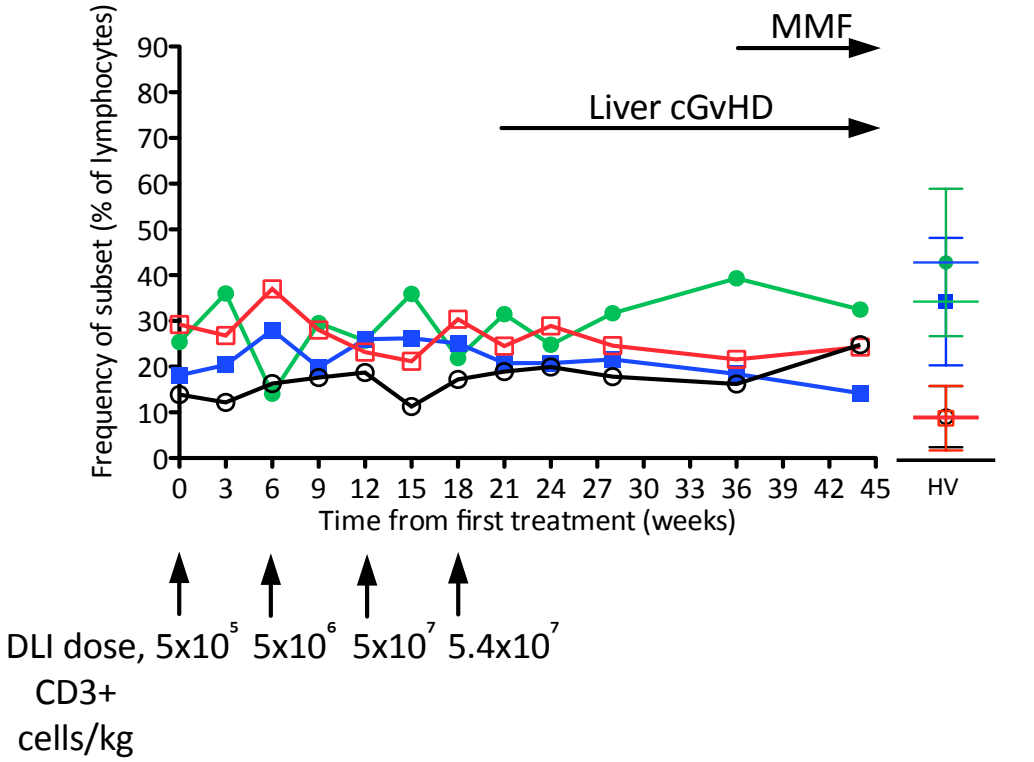
Figure 5-8 CD8+ T-cell subset composition in UPNs 14 and 22 is similar to healthy volunteers.



CD8+ T-cell subset composition was determined for each trial subject during treatment and follow-up. Results for the recipient of DLI and ACV, UPN 22, are shown on this page, and those for the two recipients of DLI alone (UPNs 14 and 32) are on the following page. Mean and standard deviation of CD8+ T-cell subset frequencies in 11 age-matched volunteers is shown in each plot.

Figure 5-8 continued

UPN 14



UPN 32

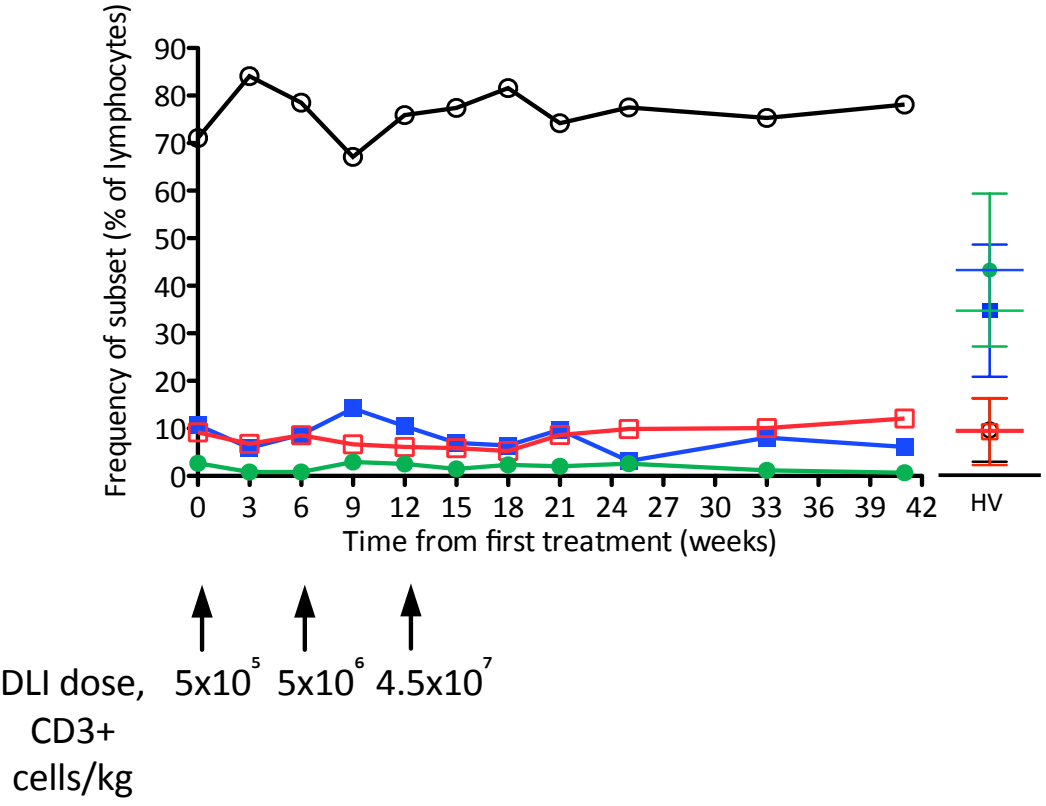
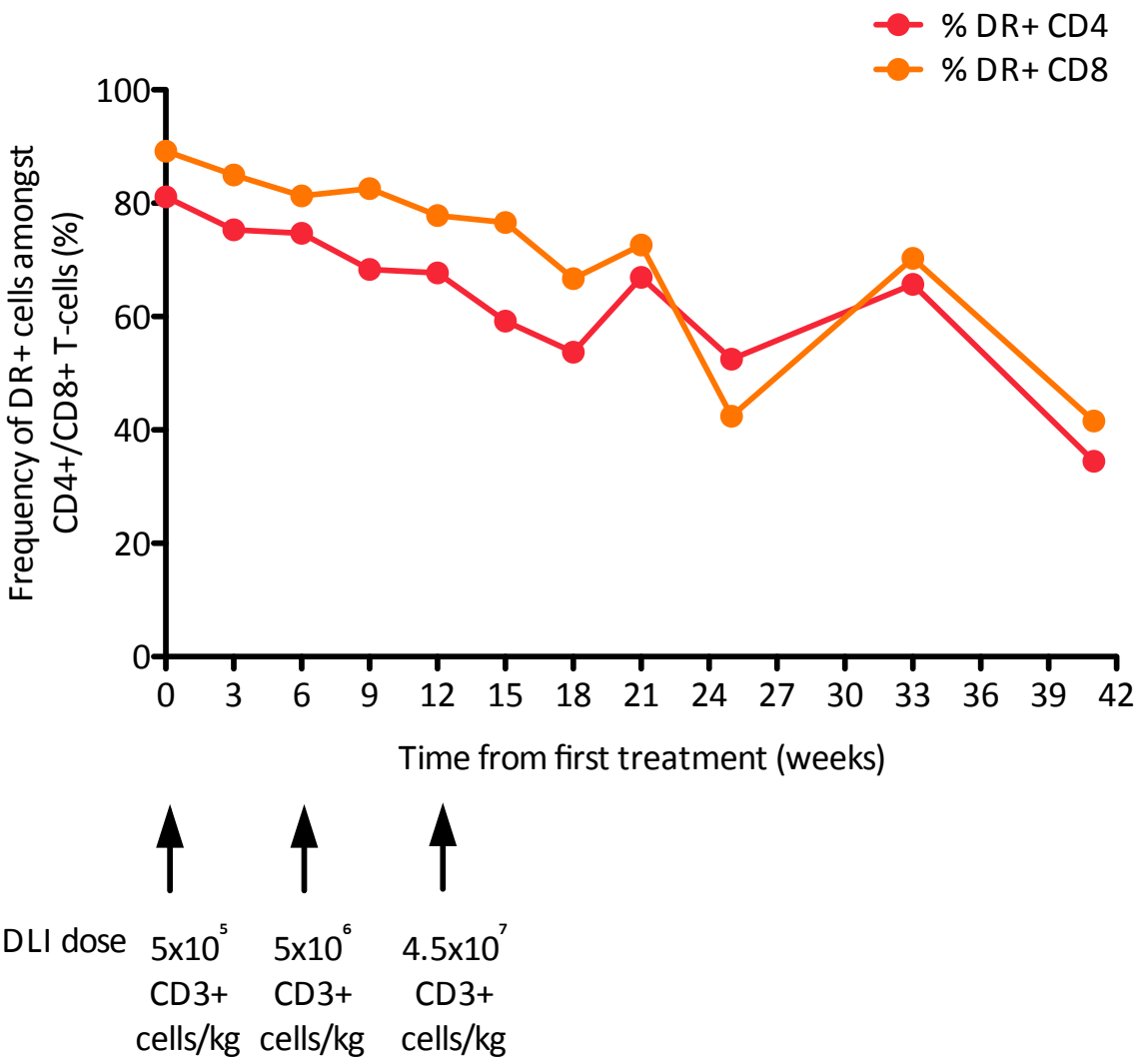


Figure 5-9 CD4+ and CD8+ T-cells in UPN 32 showed an activated phenotype at baseline that waned during follow-up



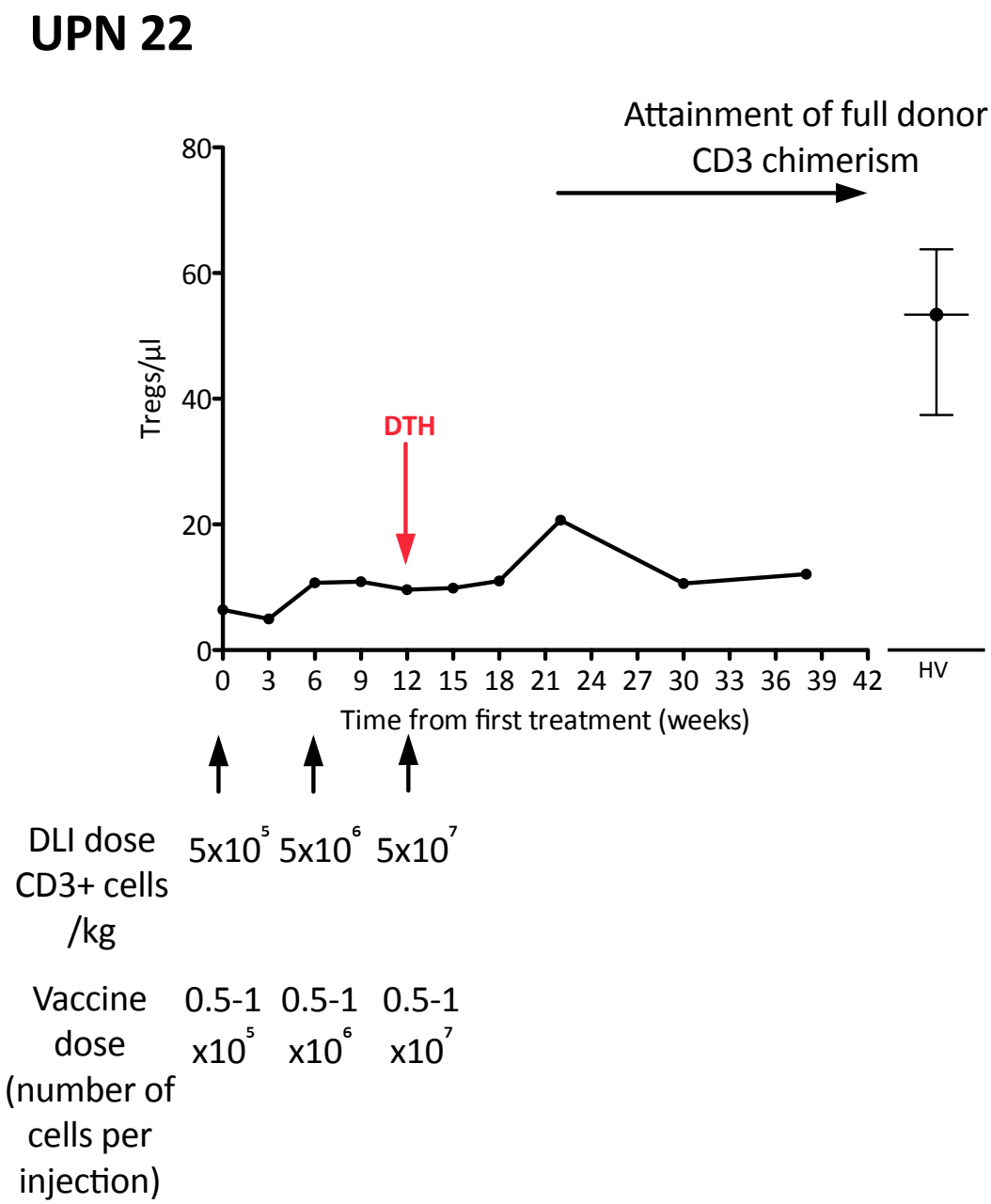
The frequencies of CD4+ and CD8+ T-cells that were HLA-DR positive at sequential time points were measured in UPN 32.

5.2.4.4 No expansion of peripheral blood regulatory CD4+ T-cells is observed following administration of DLI and ACV.

An important CD4+ T-cell population in the context of the ACV is the regulatory T-cell (Treg) subset. The secretion of interleukin-2 by the CD80/IL-2 modified AML vaccine could theoretically promote expansion of Tregs with the capacity to suppress anti-leukaemic effector functions. However, our previously published *in vitro* data suggested that whilst the absolute numbers of Tregs rose in some co-cultures containing CD80/IL-2 modified blasts and PBMCs, lysis of AML blasts was not inhibited[261]. Sequential analysis of Tregs, defined as CD27+ FoxP3+ CD25+ CD4+ T-cells, in the peripheral blood of UPN 22 revealed no change in numbers because of treatment (Figure 5-10). Expression of CD27 on CD4+ CD25+ FoxP3+ cells was used to distinguish Tregs from activated CD4+ effectors that transiently express FoxP3+ but are CD27 negative[321,322].

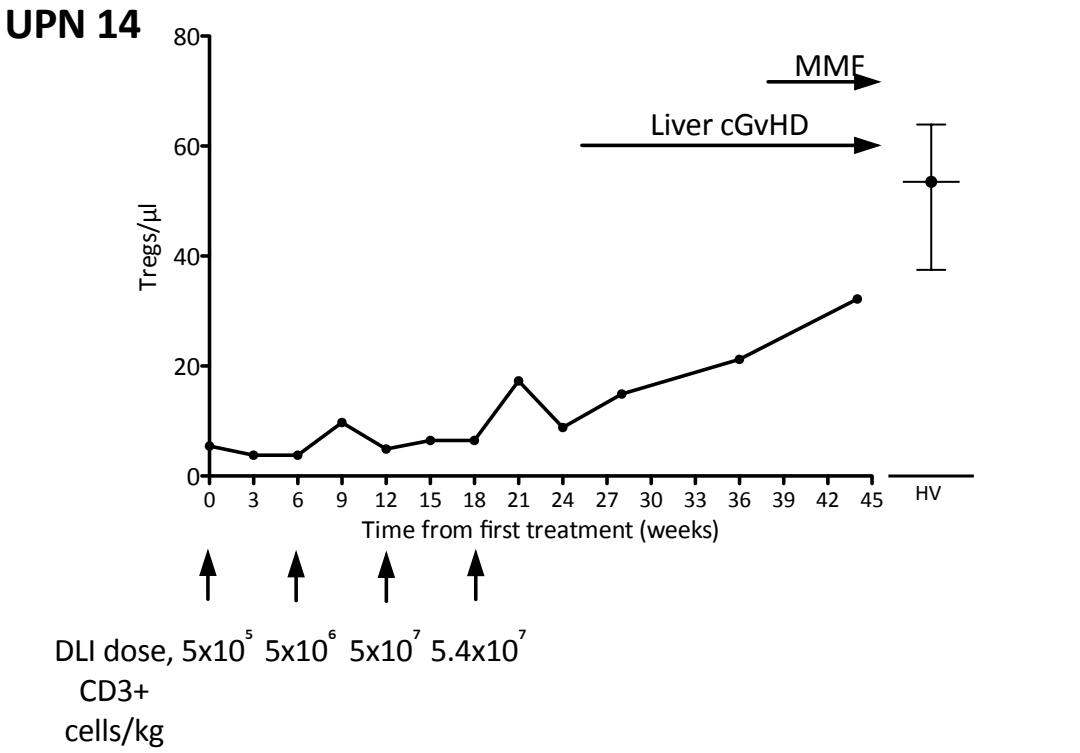
The Treg population was markedly reduced in size relative to age-matched controls in all 3 patients during follow-up, likely reflecting the low numbers of CD4+ T-cells after lymphodepleted HSCT. However, the mean frequencies of Tregs amongst the CD4+ T-cell population in UPNs 14, 22 and 32 were 5.5%, 8% and 2.6% respectively, which were similar values to the mean frequency observed in the healthy volunteers (5.3%, ranging from 3-8.2%). There was no evidence of expansion of CD25 (IL-2Rα)-bearing T-regulatory cells despite high levels of IL-2 in the serum of UPN 32. Of note, a sustained rise in absolute Treg numbers was seen in UPN 14 from approximately week 28 onwards, temporally associated with the onset of liver cGvHD in this patient. A rise in Treg numbers in peripheral blood following the onset of cGvHD has been previously observed in patients developing cGvHD following a similar RIC regimen that used alemtuzumab for lymphodepletion, (whereas anti-thymocyte globulin was used for lymphodepletion in UPN 14) [271,323].

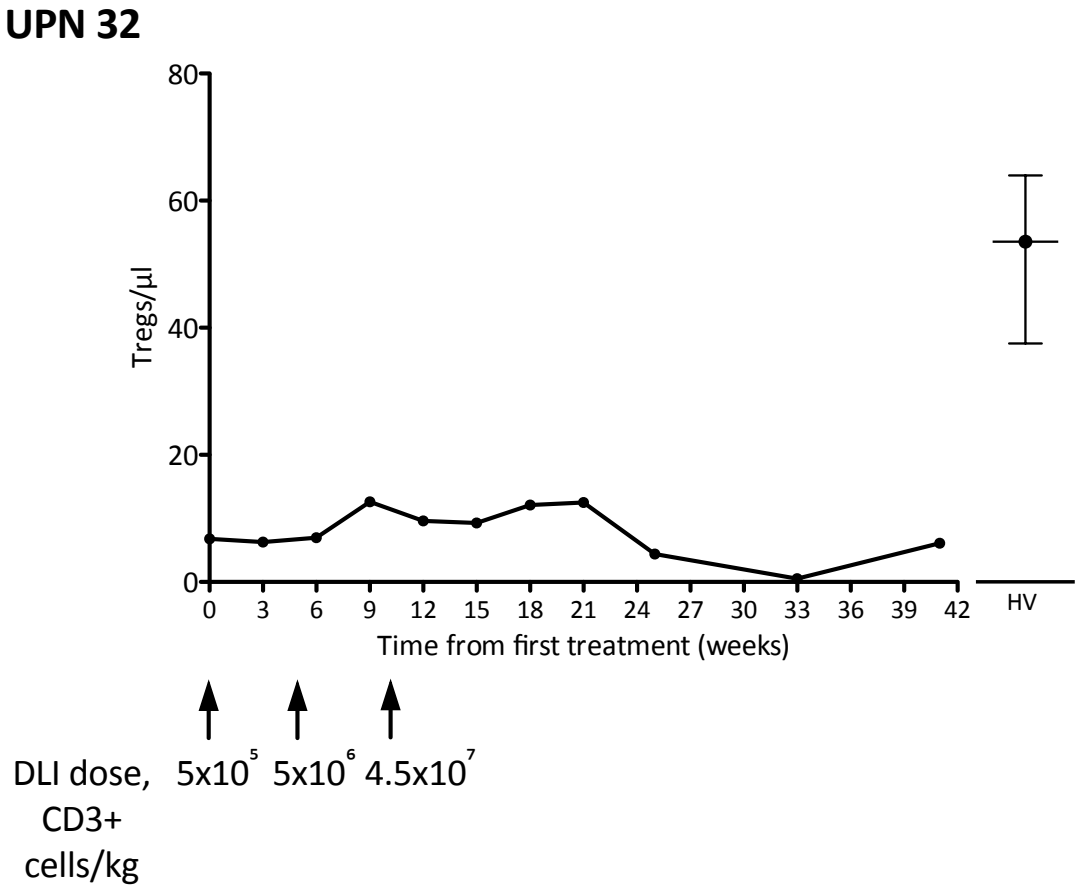
Figure 5-10 No expansion of peripheral blood regulatory T-cell numbers in UPN 22 following DLI and ACV administration



Absolute numbers of CD4+ CD25+ FoxP3+ CD27+ regulatory T-cells in the recipient of DLI and ACV, UPN 22, are shown on this page and in the two recipients of DLI only (UPNs 14 and 32) on the following page. Mean and standard deviation of Treg numbers in 11, age-matched health volunteers (HV) are shown in each plot.

Figure 5-10 continued



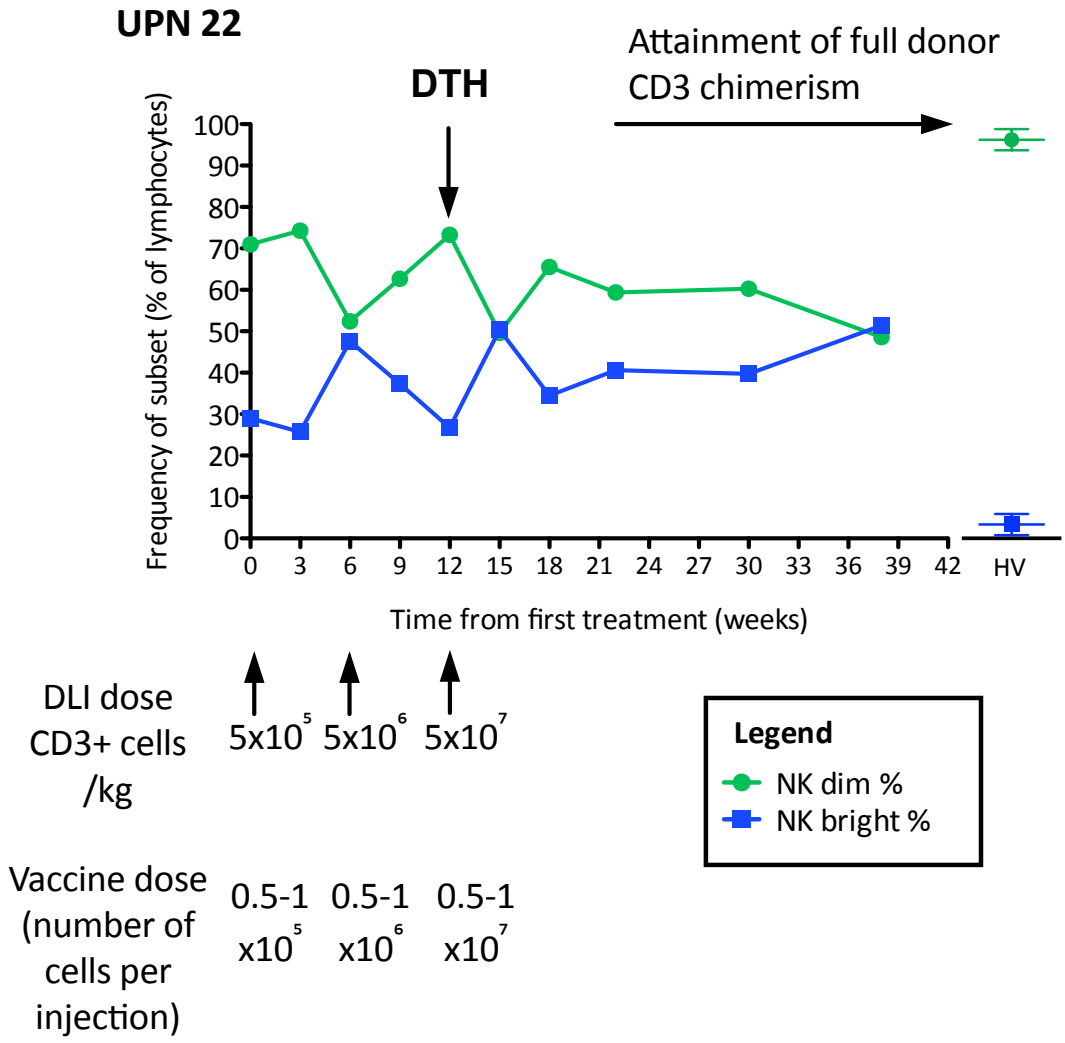


5.2.4.5 Higher than normal proportions of CD56 "bright" NK-cells observed in UPNs 22 and 32

Previously published *in vitro* data demonstrated that not only T-cells but also NK-cells could be activated more effectively by CD80/IL-2 modified AML blasts compared with unmodified blasts, resulting in superior lysis of primary unmodified AML targets[121]. Furthermore, IL-2 is capable of inducing NK-cell proliferation. Indeed, up-regulation of a selection of NK-cell activation receptors (DNAM1, NKp44 and NKG2D) was described following exposure of NK-cells to CD80/IL-2 modified blasts in the *in vitro* experiments[121]. Therefore, the number and activation status of peripheral blood NK-cells was assessed during the course of vaccine therapy and/or DLI by a sequential analysis of the major NK-cell populations in the PB of trial subjects during follow-up.

Figures 5-5 and 5-6 show the analyses of NK-cell frequencies and numbers within UPNs 14, 22 and 32 at trial monitoring time points. Absolute CD56+ NK-cell number and frequency were highest throughout follow-up in UPN 14, remaining within the range exhibited by the healthy volunteers, but were below the normal range in UPNs 22 and 32. The numbers of CD56+ NK-cells in the peripheral blood in each of the 3 patients showed no sustained changes during the course of DLI treatments. Figure 5-11 shows the NK-cell subset analyses, separated into CD56 "bright" and CD56 "dim" populations, for the trial subjects and healthy volunteers. A high proportion of CD56 "bright" NK-cells relative to healthy volunteers was observed in UPNs 22 and 32, whereas the NK-cell subset composition in UPN 14 closely resembled that of the healthy volunteers. These differences remained unchanged during and after ACV and/or DLI administration. The increased frequency of CD56 "bright" NK-cells in UPNs 22 and 32 is consistent with previous reports of NK-cell subset composition post-HSCT[324,325] and was unaltered by DLI with/without ACV. No changes to expression of the activation markers DNAM1, NKG2D and NKp44 on peripheral blood CD56+ NK-cells were observed during follow-up in any of the three patients (data not shown).

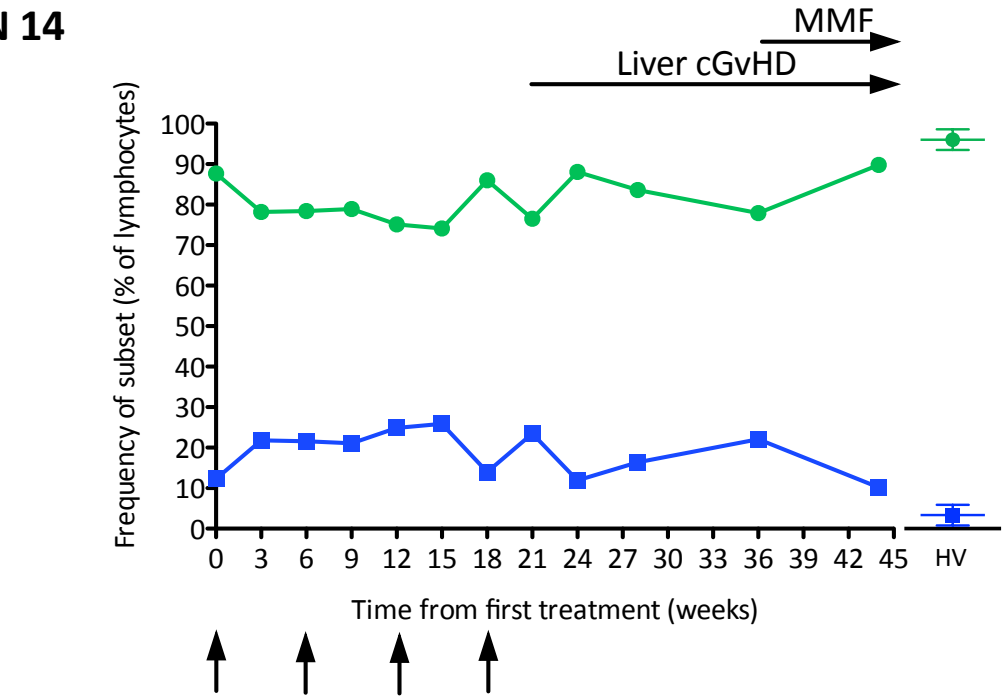
Figure 5-11 Increased frequency of CD56 “bright” NK-cells in trial subjects in comparison to healthy volunteers.



Results of sequential analysis of NK-cell subsets ($CD56_{bright}$ and $CD56_{dim}$ populations) during treatment and follow-up time points are shown for UPN 22 on this page and for the two recipients of DLI only, UPNs 14 and 32, on the following page. Mean and standard deviation of the subset frequencies are shown in each plot for 11 healthy age-matched volunteers.

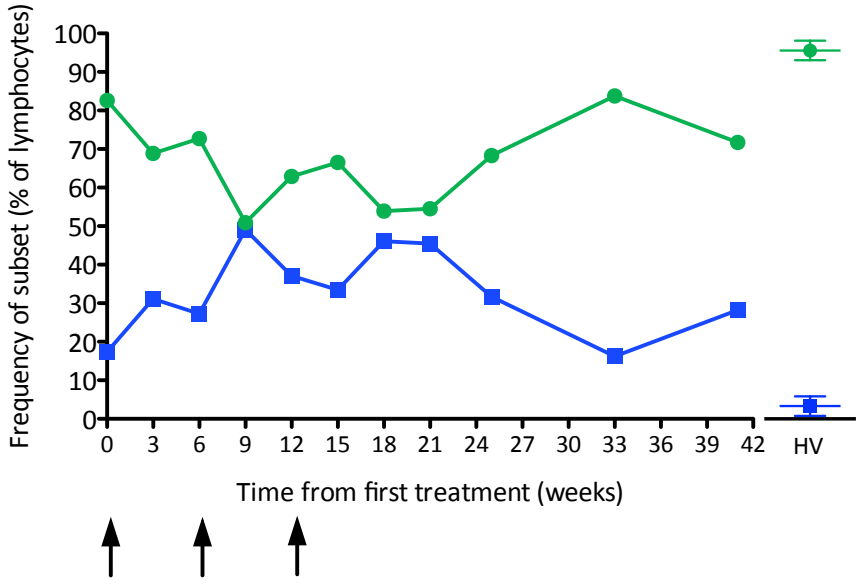
Figure 5-11 continued

UPN 14



DLI dose, CD3+ cells/kg: $5x10^5$ $5x10^6$ $5x10^7$ $5.4x10^7$

UPN 32



DLI dose, CD3+ cells/kg: $5x10^5$ $5x10^6$ $4.5x10^7$

5.2.5 TCRβ repertoire analysis in trial subjects

An important factor likely to affect the efficacy of the AML cell vaccination is the diversity of the TCR repertoire in these patients. A narrowed TCR repertoire might possess few leukaemia-reactive T-cells and therefore represent a poor responder population for activation by the ACV. DLI is administered as part of the trial protocol with the aim of increasing T cell numbers and repertoire diversity.

Adaptive Biotechnologies, Seattle, provide a commercial high throughput sequencing (HTS) assay service for analysis of the TCRβ CDR3 region using genomic or cDNA as a sensitive means to evaluate diversity of the repertoire. The technique also has utility for tracking changes in specific clone frequencies at sequential time points[275,326]. Genomic DNA samples were sent to Adaptive Biotechnologies for analysis of TCRβ repertoire diversity in UPNs 14 and 22. These 2 patients were chosen for initial study as representatives of each trial arm. Furthermore, they were both treated and studied at similar duration after HSCT. Samples from selected trial monitoring time points were sent for these preliminary investigations. Sampling time points were chosen to broadly cover the period prior to and following DLI treatment to investigate whether any changes to TCR repertoire diversity were observed after infusion of donor T-cells. The number and frequencies of productive (in-frame) unique CDR3 sequences were determined and used to derive a measurement of T-cell clonality at each time point (Figure 5-12). Individual unique productive CDR3 sequences were tracked to assess any changes in frequency during and after treatment (Figure 5-13).

The number of sequence reads per patient ranged between $0.95x10^6$-$1.8x10^6$ for the 4 samples analysed for UPN 14 and between $0.36x10^6$-$1.1x10^6$ for the 7 analysed from UPN 22 (Table 5-3). Such sequencing depths were sufficient to allow an estimation of repertoire diversity. Productive (in-frame and lacking a stop codon) uniquely rearranged TCRβ CDR3 regions sequences were identified using ImmunoSEQ analysis software (Adaptive Biotechnologies) for each sample based on the accepted IMGT consensus definitions[327]. The number of productive unique sequences ranged from 5,837-13,436 for UPN 14 and 7,741-15,091 for UPN 22 (Table 5-3 and Figure 5-12). Recent published data using the same technology

detected a median of 629,606 productive unique TCRβ CDR3 sequences (range: 58,009 to 1,161,823) in healthy stem cell donors[328].

Therefore, the 2 trial subjects demonstrated a lower number of unique productive CDR3 sequences than younger, healthy donors both before and after treatment, likely due to the T-cell depleted transplant regimen that these patients underwent.

Table 5-3 Summary of TCRβ sequencing data derived from analyses using the ImmunoSEQ analysis tool (www.immunoseq.com) at the sampling time points studied in UPNs 14 and 22.

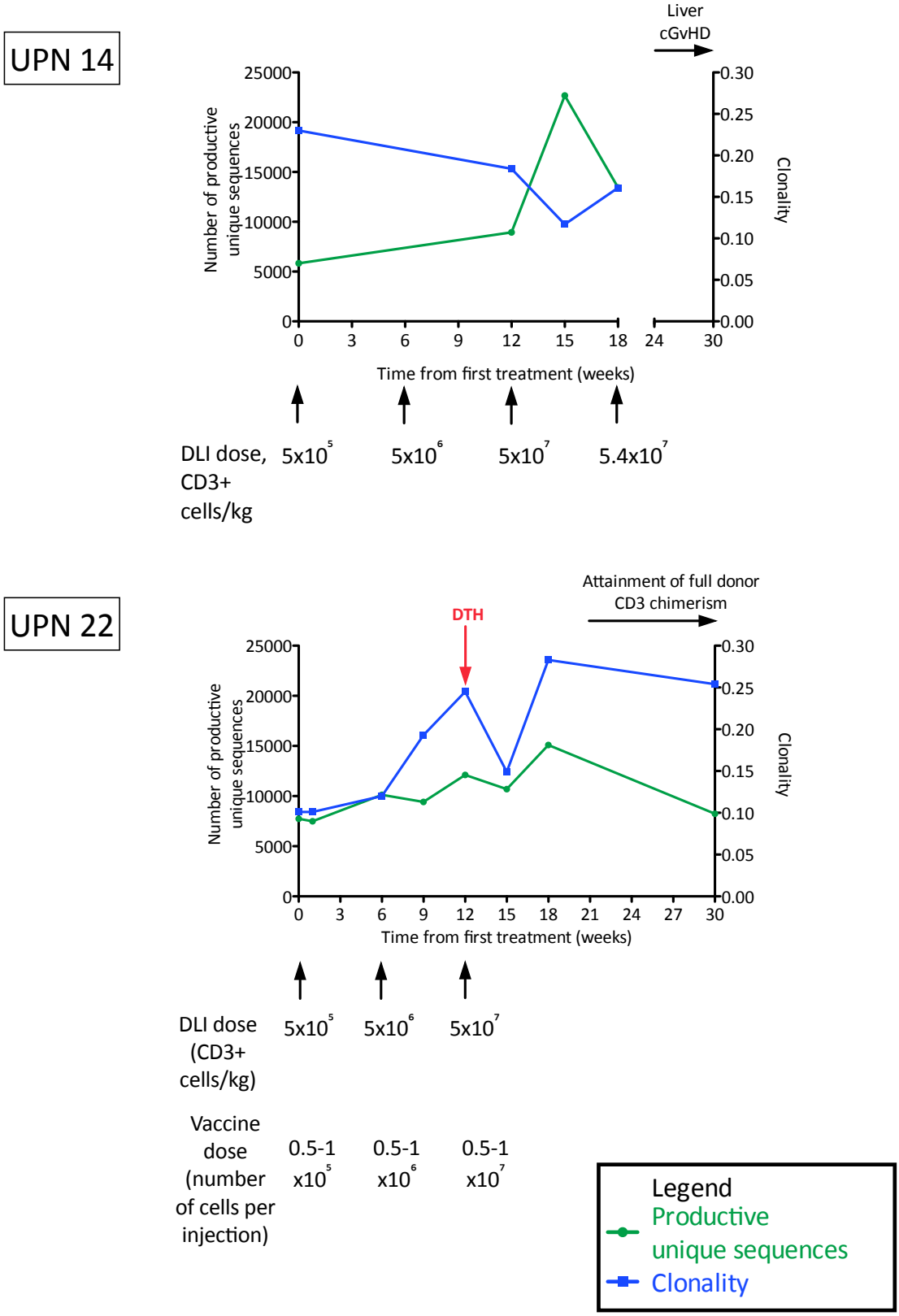
Subject and time point	Total no. of reads	No. of unique sequences	No. of productive sequences	No. of productive, unique sequences	Total no. of OOF* sequences	No. of OOF unique sequences	Total no. of sequences with stop codon	No. of unique sequences with stop codon	Entropy	Normaliz-ed entropy	Clonality
UPN 14 week 0	1182631	7116	920620	5837	131344	1159	130667	120	9.627	0.770	0.230
UPN 14 week 12	1806476	10935	1439403	8935	223003	1810	144070	190	10.711	0.816	0.184
UPN 14 week 15	1750522	27189	1427421	22674	232423	4108	90678	407	12.776	0.883	0.117
UPN 14 week 18	946948	16150	745856	13436	120585	2365	80507	349	11.507	0.839	0.161
UPN 22 week 0	1002403	9843	778592	7741	206858	1945	16953	157	11.616	0.899	0.101
UPN 22 week 1	441686	9387	340158	7490	93662	1737	7866	160	11.571	0.899	0.101
UPN 22 week 6	369326	12718	282680	10124	80083	2385	6563	209	11.708	0.880	0.120
UPN 22 week 9	794699	11965	589854	9418	191129	2353	13716	194	10.657	0.807	0.193
UPN 22 week 12	901840	15460	694975	12111	194224	3071	12641	278	10.242	0.755	0.245
UPN 22 week 15	356177	13495	272563	10696	76577	2550	7037	249	11.396	0.851	0.149
UPN 22 week 18	1091438	18949	905590	15091	165406	3511	20442	347	9.953	0.717	0.283
UPN 22 week 30	491028	10391	405793	8241	78773	1973	6462	177	9.699	0.746	0.254

*OOF, Out of frame. See methods for description of entropy calculations.

An assessment of repertoire diversity was made using a modification of Shannon's entropy (Table 5-3). Entropy is a commonly used metric of diversity that increases with both the number of elements (unique sequences) and as the evenness (in frequency) increases. For a given number of sequences, diversity is maximized when all sequences are equally abundant. As entropy is sensitive to the number of sequences considered, to account for variation in sequencing depth, entropy was normalized by the log2 of the number of productive unique sequences. Clonality was then defined as the inverse of normalized entropy to provide a metric varying between 0 and 1. For the purpose of characterizing the immune repertoire, a 0 value is returned when all sequences are equally abundant and 1 when a single sequence makes up the entire sample (see methods).

Figure 5-12 depicts the results of serial analyses of the number of unique productive TCRβ sequences and clonality for UPNs 14 and 22 listed in Table 5-3. The increasing number of unique productive CDR3 sequences observed during the course of DLI administration in UPN 14, was associated with a reduction in clonality, suggesting an increase in TCR repertoire diversity after DLI. No samples beyond the last dose of DLI have thus far been analysed for UPN 14 and therefore changes to the repertoire size and clonality during the onset and therapy of cGvHD are unknown. A small increase in the numbers of unique productive sequences was observed in UPN 22 after the second and third doses of DLI and ACV, before falling at the end of follow-up to levels seen at the start of treatment. In contrast to UPN 14, UPN 22 showed a progressive increase in clonality during treatment and follow up.

Figure 5-12 TCRβ repertoire diversity increased during treatment in both UPNs 14 and 22



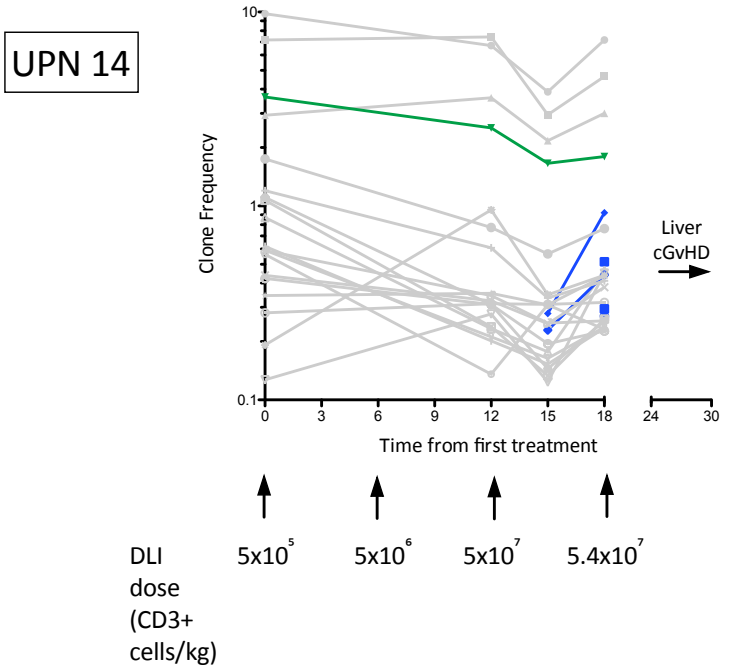
The number of unique productive (in-frame, stop codon lacking) TCRβ CDR3 sequences was determined for each patient at each sampling time point (green line). Clonality (derived using a measure of Shannon's entropy) at each sampling time point is shown in blue.

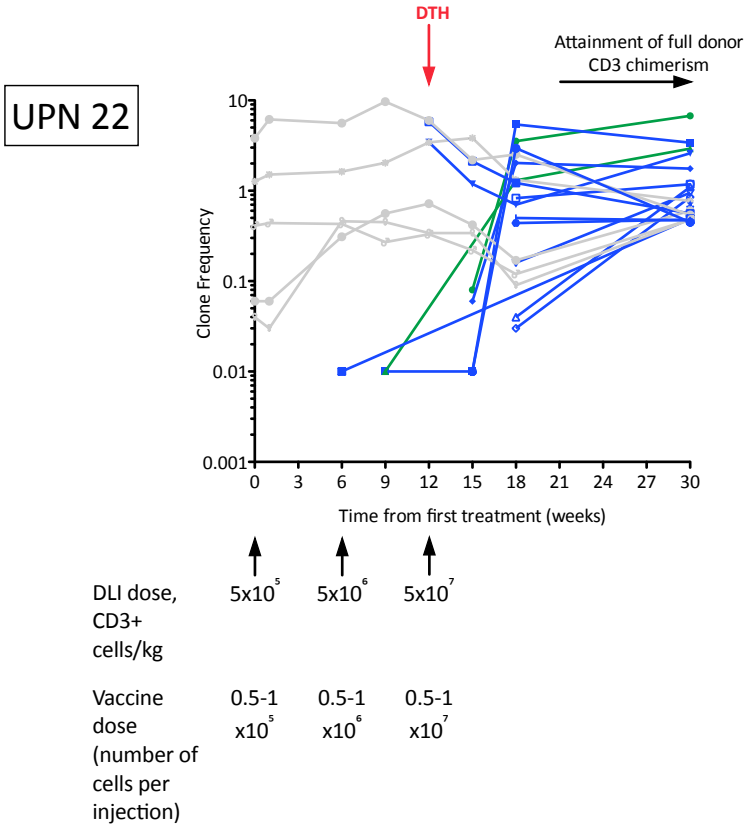
To investigate the increase in clonality observed in UPN 22 by week 30, the most abundant TCRβ CDR3 sequences representing the dominant T-cell clones at the last follow up time points were tracked back through earlier time points. Tracking of the most abundant clones detected at week 18 was also performed for UPN 14. The tracked frequencies of the top 25 clones at the last follow up point for both patients are shown in Figure 5-13.

The top 25 clones in UPN 14 at week 18 were present at consistently high frequencies during and after treatment, indicating stability of the repertoire. Only 3 of the 25 top clones at week 18 were not previously detected at week 0 in this subject. Of note, UPN 14 also showed stable, full donor T-cell chimerism prior to receipt of DLI, which persisted during the course of therapy and follow-up.

By contrast, there was a complete change in the repertoire of the dominant clones in UPN 22 during treatment. None of the 10 most abundant clones in UPN 22 at week 30 were detected at week 0 prior to DLI. Most of these clones began to emerge from week 15 and rapidly increased to supersede the dominant clones present before treatment. The time-course of the emergence of these clones suggests that they were introduced with the DLI and underwent extensive proliferation, perhaps in response to antigen stimulation. This timing also coincides with conversion to full donor T-cell chimerism in UPN 22, which was detected from week 22 onwards. In fact, the last donor T-cell chimerism assessment was 7 weeks prior to that, at week 15, therefore conversion to full donor T-cell chimerism could have taken place at any time between weeks 15 and 22, indicating that dominant T-cell clones arising from this time period were of donor origin.

Figure 5-13 Tracking the frequencies of the top 25 TCRβ CDR3 clones present at either week 18 in UPN 14 or week 30 in UPN 22.





Frequencies of individual clonotypes identified by unique productive CDR3 sequences as a proportion of the total reads at weeks 18 and 30 in UPNs 14 and 22 respectively were determined. Each clonotype was then sorted in order of frequency and their prior frequencies tracked to investigate any changes in frequencies during follow-up. Clones that were present at week 0 are coloured in grey. Clones that became detectable at later time points are coloured blue. The public EBV antigen-specific clonotypes CSVGTGGTNEKLFF and CSVGSGGTNEKLFF that are among the top 25 clones at the last time point assessed in both UPNs 14 and 22 are highlighted in green. Note log scale.

The CDR3 amino acid sequences of the top 10 clones at week 30 in UPN 22 were predicted from the nucleotide templates (Table 5-4). No obvious shared pattern or motif is observed among the CDR3 sequences, furthermore it is neither possible to imply or exclude from these sequences that recognition of similar targets by these clones has occurred. Interestingly, two of the sequences are known public clonotypes previously identified in T-cells specific for HLA-A*02:01 binding the immunodominant EBV-derived peptide GL9 (a nonamer sequence, $GLCTLVAML_{259-267}$, from within the early lytic protein BamHI-M leftward reading frame 1, BMLF1)[329,330]. Such public TCR clonotypes comprise residue-identical CDR3 sequences with the same Vβ and Jβ gene usage. They occur repeatedly within multiple individuals sharing a specific MHC-type. Public TCR clonotypes have most frequently been reported in the context of certain infectious diseases (such as EBV, CMV) but are now increasingly being identified in other pathological states such as malignancy and autoimmune disease[331].

Table 5-4 Amino acid sequences and V and J gene usage of the CDR3 regions of the 10 most abundant clones at week 30 in UPN 22 (unique productive sequences only)

Amino acid sequence	Variable (V) beta-chain gene	Junctional (J) beta-chain gene	Frequency of all reads (%)
CSVGSGGTNEKLFF*	*TRB V29-1*	*TRB J1-4*	6.75%
CASYLGRGIGDEQYF	*TRB V11-2*	*TRB J2-7*	3.40%
CSVGTGGTNEKLFF*	*TRB V29-1*	*TRB J1-4*	2.92%
CASGTEAFF	*TRB V12-5*	*TRB J1-1*	2.60%
CASSKAGQGGDTEAFF	*TRB V9-1*	*TRB J1-5*	1.19%
CASSLASRVGTDTQYF	*TRB V5-1*	*TRB J2-3*	1.18%
CASSLGVSQPQHF	*TRB V12-3/12-4*	*TRB J1-1*	0.85%
CASSMGGNYGYTF	*TRB V9-1*	*TRB J1-2*	0.72%
CASGRWNNEQFF	*TRB V12-5*	*TRB J2-1*	0.64%
CASSPILGDTEAFF	*TRB V3-1*	*TRB J1-1*	0.62%

*public clonotypes that recognise GL9/A2*01

CDR3 sequences, Vβ and Jβ identities matched using the international IMmunoGeneTics (IMGT) database[278,332]; TRB, T-cell receptor β-chain

To put our observed clonotype frequencies into context, a recently published study of TCR repertoire recovery after HSCT, using 5' rapid amplification of cDNA ends PCR, found that 19 clonotypes at frequencies >1%, were observed in a patient at 138 days post-HSCT, with the highest clonotype frequency reaching 11%[333]. By contrast, individual clonotypes rarely accounted for more than 1% of clones in healthy volunteers. One of the dominant clonotypes identified by this group was the same public clonotype CSVGSGGTNEKLFF observed in our study, which became prominent during an episode of EBV reactivation in that patient[333].

UPN22 is EBV antigen positive and therefore harboured latent virus within tissues. The two HLA-A*02:01 restricted EBV specific public TCR clonotypes were not detected in UPN 22 prior to receipt of DLI. The donor of UPN22 was seronegative for EBV IgG indicating that the EBV specific T-cells that expanded rapidly after DLI likely emerged from the naïve T-cell population within the DLI upon exposure to EBV antigen in the patient. Emergence of new clonotypes after DLI suggests that delayed add back can replenish the T-cell repertoire. Screening for EBV reactivation by EBV PCR analysis on peripheral blood in UPN 22 was negative at all trial time points prior to week 22, at which point a low copy number of 446 copies/ml was detected. EBV remained detectable (between 92-771 copies per ml) until the last monitoring time point in this patient. Prior to study entry, UPN22 had experienced intermittent low-level fluctuations in EBV copy number of similar magnitude in their peripheral blood (data not shown). Expansion of EBV specific clonotypes preceded the detection of EBV by PCR in the peripheral blood of the recipient during the trial monitoring period. Given that EBV antigen would be present within the tissues of the recipient, it is conceivable that expansion of EBV-specific clones could occur without detectable EBV in peripheral blood.

By comparison, the EBV specific clonotype CSVGTGGTNEKLFF was observed in UPN 14, who is also HLA-A*02:01+, at a frequency of 1.8% at week 18 and this clone was detected before administration of DLI, at week 0 (Figure 5-13). UPN 14 and their donor were both known to be EBV antigen positive. Furthermore UPN 14 had full donor T-cell chimerism at the time of receiving the first DLI. EBV was first detected at week 3 in the peripheral blood of UPN 14, at which point a very low copy number of 92 copies/ml was

found. EBV then became transiently detectable at week 24 when 918 copies/ml were detected, before becoming undetectable over the remainder of follow-up on trial. This subject therefore showed no dramatic clonal turnover following DLI or change in donor T-cell chimerism and showed stability of an EBV-specific clonotype through treatment. Since UPN 14's donor had previously been exposed to EBV, by contrast to UPN 22's donor, stimulation and expansion of naïve donor T-cells would not be expected to occur.

These preliminary data show that although TCR repertoire diversity is reduced in trial subjects compared with healthy donors, responses to antigens such as EBV emerge after DLI administration. A number of other clonotypes were also only detected in UPN 22 following DLI administration and underwent rapid expansion. At present, their antigen specificity remains unknown. However, these findings suggest that DLI may also enable responses to other antigens, such as epitopes within the AML cell vaccine.

5.3 Discussion

These data describe a novel approach to improve the unsatisfactory long-term responses observed in patients treated with therapeutic DLI for relapsed acute myeloid leukaemia. This is the first study to combine a patient-specific, gene-modified whole AML cell vaccine with an immunotherapeutic platform of DLI following salvage chemotherapy for relapsed AML, with the goal of generating a durable and effective GvL response. The use of a whole cell vaccine enables presentation of a range of both defined and undefined leukaemia-associated antigens to the donor immune system. These include haematopoietic-cell restricted mHags capable of stimulating alloreactive GvL responses in donor T-cells. Exposure to a range of LAAs within the ACV has the potential advantage of avoiding induction of escape mutants. A self-inactivating, fourth generation lentiviral vector which was deleted of all viral genes (3' LTR deletion) has been successfully employed to prioritise safety of this approach by virtually eliminating the risk of generating replication competent lentivirus.

The single patient, UPN 22, who received 3 doses of ACV, demonstrated no vaccine-related adverse events. No evidence of replication competent lentivirus was observed in either of the 2 patients treated in the vaccine and DLI arm. The primary endpoint of this Phase I study is safety and although the trial is ongoing, absence of RCL generation post-injection, using a combination of ELISA and PCR analyses for detection, is highly encouraging and consistent with reports in the literature suggesting the safety of this approach[334,335]. A single dose-limiting toxicity, due to an expected complication of DLI administration, namely cGvHD, was observed in UPN 14 (who received DLI alone). No clinical evidence of vaccine-induced autoimmune toxicity was observed in UPN 22 although a transient detection of anti-smooth muscle antibodies was detected during their treatment phase. Anti-nuclear antibodies were also found in the sera of UPNs 14 and 32 in the DLI only arm. *De novo* detection of naturally occurring autoantibodies has been reported in recipients of allogeneic HSCT[336]. Autoantibodies have also been reported in patients with cGvHD, including specifically anti-nuclear antibodies, similarly to UPN 14[337]. Whether this phenomenon was present in these patients at earlier time-points pre- or post-HSCT is unknown, given that these tests are not part of routine follow-up. The time-course of autoantibody detection (and restricted detection in UPNs 14, 22 and 32 but not UPN 13, who received only a single dose of DLI and ACV) raises the possibility that infusion of DLI may have triggered autoantibody generation. This in turn could suggest infusion of

CD4+ T-cells in DLI to recipients may provide helper function to activate B-cells[338]; alternatively epitope spreading as a consequence of tumour cell lysis by donor lymphocytes might be a further means to induce such antibodies. Importantly these observations also highlight that screening for autoantibodies may not reliably discriminate vaccine-specific autoimmune complications from DLI-related sequelae.

The most compelling observation so far suggestive of an immune response to vaccination in UPN 22 has been the development of a DTH response following dose 3 of ACV and DLI. Formal DTH response evaluation measures antigen-specific immune recall in cancer immunotherapy trials by re-challenging immune cells to an immunogen administered at a prior vaccination[252]. DTH is defined by observation of induration and erythema at vaccination sites persisting at 48-72 hours following re-exposure; biopsy of these sites typically reveals lymphocytic and granulocytic infiltration with accompanying evidence of increased vascular permeability[252]. This has been shown in an animal model to typically comprise a CD4+, Th1-mediated recognition of peptides presented in the context of MHC Class II molecules[339]. The vaccination site biopsy in UPN 22 did indeed contain a predominantly T-lymphocytic infiltrate. Unlike the peripheral blood, a more normal CD4:CD8 T-cell ratio of 2:1 was observed by immunohistochemistry (data not shown), suggesting this represented a true DTH reaction. Development of DTH reactions at vaccination sites was observed in long-term responders following the most comparable gene-modified whole cell vaccination strategy to ours reported to date, by Ho and colleagues. In this study, 15 patients with AML/MDS were vaccinated up to 6 times between day 30 and 100 post T-replete HSCT with an autologous vaccine genetically modified using an adenoviral vector to express granulocyte-macrophage colony stimulating factor (GM-CSF) as an immune adjuvant[246]. All patients had persistent morphological excess of blasts at transplant and therefore were at high risk of relapse. Seven of 8 patients who underwent formal histological assessment for a DTH response following receipt of the fifth dose of vaccine demonstrated histological changes compatible with development of a DTH reaction. These patients also remained in remission at the time of trial reporting, with a median of 26 months follow-up[246].

Some[340-342], but not all [343,344], investigators have shown that development of DTH responses correlates with clinical and/or immunological outcomes following vaccination. Differences in observations are partly attributable to the variety of vaccination approaches and lack of standardised methods for measuring DTH responses between studies. A more detailed analysis of antigen-specific responses at DTH sites using *in situ* staining of biopsy tissue with fluorescently labelled tetramers has been used to dissect target peptide recognition by infiltrating T-cells following melanoma vaccination[345]. Additionally, T-cells were isolated from biopsy sites and following a brief culture period, analysed for antigen-specificity *in vitro*[345]. An attempt at culturing T-cells from a skin biopsy site from UPN 22 was made, but was unfortunately unsuccessful. This may in part be due to the late collection of the skin biopsy at more than 72 hours post-vaccination, resulting in a relatively lower number of infiltrating lymphocytes available for culture. However, high-throughput sequencing of DNA obtained from these biopsies is planned. Given the emergence of new T-cell clones in the peripheral blood after the DTH response in UPN 22, it will be of great interest to determine whether TCRβ sequences present at biopsy sites are among those that emerged and expanded in PB after DLI with ACV. This finding would suggest that leukaemia-specific T-cell clones have been stimulated in response to therapy.

Formal assessment of DTH was not described in the original trial protocol. Following the experience with UPN 22, I have written a modification to the clinical trial protocol which describes a planned administration of 2 control injections with each vaccination containing irradiated, unmodified tumour cells in one injection and carrier medium alone in another. This should distinguish generation of non-specific reactions against components in the medium from specific responses to cellular component. The Medicines and Healthcare products Regulatory Agency (MHRA) and relevant ethical bodies have approved this amendment, which will be used to assess patients recruited to the vaccine arm in future.

Analysis of lymphocyte numbers in the trial subjects revealed that all 3 patients had demonstrated some recovery from their lymphopenic states early post-HSCT and that their lymphocyte numbers were near normal, or in the normal range at the time of relapse. UPN22 developed a profound lymphopenia following fludarabine-containing salvage chemotherapy that persisted at the time that ACV administration

and DLI commenced. Immediately following recovery from the same salvage chemotherapy regimen, UPN 32 had shown an increase in lymphocyte numbers to higher than that seen in healthy volunteers. Lymphocyte numbers increased during the treatment and follow-up phases after ACV and DLI in UPN 22, and DLI alone in UPN 32 but were unchanged during treatment in UPN 14. In the absence of a control arm that did not receive DLI in this study, it is not possible to attribute the observed increments in lymphocyte numbers directly to the infusion of donor lymphocytes.

Lymphocyte subset analyses demonstrated that lymphocyte composition was abnormal in all 3 patients and was not altered substantially by administration of ACV and/or DLI. The most marked deficiency was in CD4+ T-cell numbers and frequencies, consistent with reports from other groups[315,317]. Impaired reconstitution of CD4+ T-cells following lymphodepleting transplant conditioning regimens has been attributed to the consequences of thymic atrophy in adult transplant recipients[346]. Lymphocyte composition was in other respects more similar to normal in the two patients with late relapse post-HSCT, UPNs 14 and 22. Both had near normal numbers of CD8+ T-cells throughout the trial follow-up period. Whilst UPN 14 also demonstrated near normal NK-cell numbers at all time points, UPN 22 showed persisting deficiencies in NK-cell numbers during therapy. The high total lymphocyte numbers observed throughout follow-up in UPN 32 were due to a large and continuous expansion of CD8+ T-cells (predominantly terminal effectors that may have been responding to a microbial stimulus such as herpes virus reactivations or infection with *salmonella* spp.). NK-cell numbers in this patient were persistently lower than the range seen in the healthy volunteers.

Given the previously published *in vitro* data demonstrating the ability of CD80/IL-2 modified AML cells to activate leukaemia-specific cytotoxicity mediated by T- and NK-cells[121], analysis of these specific cell types within trial subjects is vital. Although absolute numbers of CD4+ and CD8+ T-cell numbers in UPNs 14 and 22 were lower than the range seen in the healthy volunteers, CD4+ and CD8+ T-cell subset frequencies, specifically proportions of CD4+ and CD8+ naïve T-cells, were close to normal in these two patients. In view of the important role naïve T-cells play in mediating alloreactive GvL responses[42,300,301], this finding is also of importance when considering whether these patients will be able to respond to therapeutic vaccination. Crucially, no systemic rise in regulatory T-cell numbers or frequency was observed in UPN 22 during follow-up, despite exposure to the IL-2 secreting vaccine. This suggests that, despite repeated

exposure to the vaccine, induction of immunosuppressive T-cell responses that could hamper leukaemia-specific cytotoxicity is unlikely.

Additionally, using a novel high throughput sequencing approach, an evaluation of the TCRβ repertoire during the course of therapy was made in two of the trial subjects. A modest rise in the number of unique productive TCRβ sequences in both UPNs 14 and 22 consequent upon DLI administration was observed. Secondly, an increase in TCR clonality in UPN 22 occurred after DLI and ACV administration. Expansion of T-cell clones bearing TCRβ sequences that are public clonotypes known to be expressed by EBV-specific CTLs was observed following DLI administration in UPN 22. This observation, that responses to the EBV immunogen emerged after DLI, lends support to the hypothesis that DLI boosts TCR repertoire diversity, allowing response to a range of stimuli, including not only pathogen-derived targets but also possibly LAAs. Several other clones also emerged after DLI and ACV in UPN 22. However, at present, it is not possible to predict the cognate epitope from the CDR3 amino acid sequence or to suggest recognition of related peptide-MHC (pMHC) complexes based on CDR3 sequence similarity alone. This is due to the high complexity of TCR/pMHC interactions and the difficulty of predicting the folding and physio-chemical properties of three-dimensional protein structures.

As yet there are no functional data directly assessing anti-leukaemic responses in PBMCs isolated from the three trial patients. These assays continue to be optimised although progress has been hampered by the low T-cell numbers in these patients and limited availability of leukaemic blasts for use as targets in assays. This is particularly an issue for UPN 22, whose stored leukaemic blasts were prioritised for vaccine production. Antigen-specific production of IFNγ and Granzyme B using Enzyme Linked Immunosorbent Spot assay (ELISpot) will be one means to assess functional activity[320]. The potential to use HTS to track T-cell clones during treatment and between vaccination, marrow and PB sites is an exciting prospect for future study. Only with analysis of additional patients, in a study powered to detect immune response as a primary endpoint, might it be possible to confidently demonstrate evidence of vaccine-induced leukaemia-specific immune responses.

In conclusion, these findings illustrate the safe treatment of a patient with ACV and DLI and evidence supporting induction of a response to vaccination in the form of a DTH reaction. The difficulties of conducting such a study are highlighted, given the large number of patients screened but recruitment rate of only 10%. Given these preliminary safety data in support of the ACV and *in vitro* evidence of induction of superior leukaemia-specific cytotoxicity in the autologous setting, I have written a clinical trial protocol to allow vaccination of AML patients who are ineligible for allogeneic HSCT. This complementary Phase I study will run in parallel to increase patient recruitment. Positive safety findings from these studies should allow progression to a Phase II trial including greater numbers of patients, where development of immunological responses can be more rigorously evaluated.

Chapter 6 Summary and Future Work

6.1 Summary

The studies presented in this thesis examined two forms of immunotherapy to treat myeloid malignancies, one that is in current clinical use and a second that is in development.

Firstly, adoptive transfer of unselected lymphocytes in the form of DLI, representing the most frequently employed means to enhance GvL after allogeneic HSCT and mediated principally by alloreactive donor T-cells, was examined. Following disease recurrence after allogeneic HSCT, DLI has shown considerable efficacy in the treatment of more indolent haematological malignancies such as chronic myeloid leukaemia or lymphoma. In the context of AML and MDS, however, there has been limited evidence to support DLI efficacy. The data presented in Chapter 3 have specifically described outcomes from a single institution following therapeutic and pre-emptive DLI in the setting of confirmed or impending relapse (suggested by declining donor/predominantly recipient T-cell chimerism) following TCD RIC HSCT for AML or MDS. I have shown that therapeutic DLI (tDLI), given alone or following disease bulk reduction by chemotherapy, is associated with an estimated 5-year overall survival of 40%, exceeding the responses reported by other groups that are predominantly in the T-replete, myeloablative HSCT setting. However, the progression/relapse rate in tDLI recipients at our institution approached 70% at 5 years suggesting that durable remissions are not currently being achieved. Therefore approaches to increase the frequency and duration of responses to tDLI are highly desirable. The best responses to tDLI were observed in patients with low disease burden prior to treatment, suggesting that DLI is most effective as therapy in the context of minimal residual disease. Furthermore, administration of pre-emptive DLI (pDLI) in the absence of overt disease recurrence resulted in excellent 5-year overall and event-free survivals of 80% and 65% respectively. Only 3 patients with chimerism improvement following pDLI subsequently relapsed. These outcomes were in direct contrast to the 100% relapse rate for patients who showed an incomplete chimerism response to pDLI. In combination, the data from both the tDLI and pDLI cohorts supports the potential for provision of durable GvL activity by infused lymphocytes. The incidence of GvHD following DLI was highest for the tDLI cohort (45%) whilst a moderate rate (31%) was observed in the pDLI recipients, indicating the difficult challenge of separating effective GvL induction from unwanted toxicity of GvHD.

The second immunotherapeutic approach examined was immunisation that aims to selectively induce T-cell responses against leukaemic blasts and thus avoid GvHD. One focus was the well-studied leukaemia-associated antigen WT1 that has been identified as a high priority target for future studies of immunotherapy, not only in the context of haematological malignancies but also in solid tumours[157]. Phase I and II studies of vaccination in AML and MDS patients using a single short peptide or multiple epitopes from this self-antigen have shown promising expansion of functional WT1-specific T-cells, temporally associated with reductions in disease burden in some cases[198,199,201]. However, the responses have generally been short-lived and of low avidity[200]. In Chapter 4, I have described pre-clinical studies of a novel combination of adjuvants (CASAC) to combine with WT1 peptide vaccination to induce WT1-specific T-cell responses in C57BL/6 mice. The inclusion of TLR agonists and activating CD40 antibodies within the vaccine aims to induce cell-mediated immunity via activation of professional antigen presenting cells (APCs). A number of these adjuvants are now entering the clinic. Repeated immunisations were well tolerated by mice. In those that responded to vaccinations, expansion of WT1-specific T-cells capable of potent *in vivo* cytotoxicity was observed. Importantly, T-cell responses were induced against the well defined MHC Class I epitope WT1-RMF using a cocktail of overlapping long peptide sequences spanning the whole WT1 protein. If translated to the clinical setting, such an approach would have the advantage of allowing a single product to be employed universally to patients irrespective of HLA-type. Furthermore, multiple Class I and II epitopes, including hitherto uncharacterised immunogenic WT1 sequences, may be simultaneously targeted. Vaccination with long peptides has the additional advantage of directing presentation to professional rather than non-professional APCs, thus avoiding potential induction of T-cell anergy[214]. However, while CASAC was shown to combine successfully with WT1 peptides to induce WT1-specific T-cell responses, these were inconsistently induced, with some mice showing no response to vaccination at all. This suggests that further efforts are required to improve response rates and magnitude.

Given the high rates of disease recurrence following tDLI reported in Chapter 3, a novel strategy combining tDLI with immunisation to augment induction and durability of GvL reactions is being evaluated in a phase I clinical trial. I described the preliminary results in Chapter 5. The vaccine used was a patient-specific whole cell vaccine derived from the patient's AML blasts, thus harbouring all relevant LAAs and

mHags to that patient. A self-inactivating lentiviral vector designed to maximise safety of this approach and eliminate the risk of inducing replication competent lentivirus in recipients was used to successfully transduce primary AML blasts collected from 2 patients with relapsed AML post-allogeneic HSCT. Two patients, UPNs 14 and 32 were allocated to receive DLI only following remission re-induction after AML recurrence. UPNs 13 and 22 were allocated to receive DLI along with the patient-specific AML Cell Vaccine (ACV). UPN 13 experienced a rapid relapse within weeks of treatment with the first dose of DLI and ACV. UPN 22 was the only subject that received 3 doses of the ACV and DLI and experienced no immediate adverse immune reactions and showed no signs subsequently of autoimmune toxicity or GvHD. Following the third vaccination, a DTH reaction was observed. Researchers have reported DTH responses to be favourably linked to sustained remissions following immunotherapy[340-342]. Analysis of peripheral blood lymphocyte numbers in the 3 trial subjects prior to and following therapy with ACV and/or DLI demonstrated that these were within or near normal range at study entry and rose during the course of treatment in 2 subjects. Lymphocyte subset composition however was abnormal in all 3 patients in comparison with age-matched volunteers. Divergence in lymphocyte subset composition from that seen in volunteers was most prominent in UPN 32, who was within six months of HSCT at the time of relapse and entry to the trial, compared with UPNs 14 and 22 who were 18 and 34 months respectively post-HSCT. Receipt of ACV and/or DLI did not substantially alter lymphocyte subset composition. However, TCRβ repertoire analyses revealed a dramatic change in one of two patients studied during therapy. Following the third dose of ACV and DLI in UPN 22, a rapid rise to dominance of previously undetected TCRβ clonotypes was observed. Of particular note, two of the clonotypes were known sequences expressed by EBV-specific T-cell clones specific for public HLA-A*02:01 restricted epitopes. UPN 22 is EBV seropositive but the donor is EBV seronegative. Emergence and rapid rise to dominance of EBV specific clonotypes after DLI suggests priming and proliferation of naïve T cells introduced by DLI has occurred. This very promising finding suggests that infusion of donor T-cells may expand the recipient T-cell repertoire, permitting responses to novel antigens, including microbial targets and by extension, potentially leukaemia-associated antigens.

The overall theme of the studies presented has been to explore methods to boost leukaemia-specific T-cell responses in AML patients. This may be achievable by infusion of naïve T-cells within donor

lymphocytes to induce responses to immunogens including LAAs. Vaccination against epitopes from LAAs such as WT1 may help induce leukaemia-specific T-cell responses, including cytotoxicity and cytokine production. Preliminary findings suggest that vaccination using genetically modified AML blasts from patients is feasible and safely combines with DLI administration. More patients need to be treated and evidence sought for emergence of leukaemia-specific immune responses post-vaccination before safety and efficacy can be clearly demonstrated. These issues are discussed in the following section describing future investigations arising from the results presented.

6.2 Future work

Data from the analyses of lymphocyte composition and the TCRβ repertoire in the small number of RFUSIN2-AML1 trial subjects studied so far raise interesting questions concerning the immunological consequences of DLI. Despite limited effects of DLI on lymphocyte subset composition, changes in T-cell clonal dominance following infusion of donor lymphocytes were observed in one of 2 patients studied. The emergence and expansion of T-cell clones specific for EBV only detectable after the third (largest) DLI and ACV dose in UPN 22 suggests that naïve donor T-cells present in the infused product may respond to antigens within the recipient. Although this was in the setting of therapeutic DLI, it is likely that a similar mechanism underlies the beneficial effects of pDLI. Taking into consideration the results of the retrospective study of outcomes following pDLI that showed improved and sustained EFS is achievable in these patients who are at high risk of relapse, it is hypothesised that the naïve T-cell repertoire is broadened within the DLI recipient and includes T-cells reactive against LAA-derived epitopes that confer protection from disease relapse. Substantiation requires prospective study on a larger scale. Furthermore, an important limitation of both the retrospective clinical study of pDLI outcomes and the analysis of lymphocyte subset and repertoire analyses in trial subjects is the absence of a comparator group that does not receive DLI. An earlier report from our institution indicated a trend towards improved relapse-free survival for recipients of pDLI compared to patients spontaneously achieving full donor chimerism (FDC) or stable mixed donor chimerism following FBC-conditioned allogeneic HSCT for AML and MDS[156]. Those data, along with the impressive OS and low incidence of relapse in patients showing improvement in donor T-cell chimerism following pDLI reported in this thesis suggest that delayed add-back of donor T-cells may confer protection from relapse.

These hypotheses would be more clearly defined by a prospective investigation of lymphocyte subset composition, function and TCRβ repertoire diversity post-TCD RIC HSCT in a large number of AML and MDS patients. In such a study, patients who do not receive DLI and age-matched healthy volunteers would serve as control groups. A detailed sequential analysis of the kinetics of donor T-cell chimerism post-HSCT combined with analysis of lymphocyte composition and function could highlight differences in recovery between patients with spontaneous development of predominant donor chimerism and those with delayed attainment augmented by pDLI. Functional assays to assess anti-leukaemic responses are also required. These would consist of killing assays, measuring lysis of the patients' primary AML blasts by matched patients' PBMCs after *in vitro* culture. Similar cultures would allow assessment of cytokine production associated with cytotoxic responses, such as IFNγ and granzyme B, in ELISpot assays. Evaluation of leukaemia-specific responses in patients that do or do not require pDLI (and also at successive time-points pre-and post-pDLI) could inform whether the observed protection against relapse afforded by pDLI correlates with enhanced leukaemia cytotoxicity *in vitro*. Serial measurement of minimal residual disease, where a molecular marker of disease such as NPM1 mutation or WT1 over-expression is detected in a patient, could provide a sensitive reading of disease burden in the absence of overt relapse in these patients. Given that other groups have reported the emergence of T-cells specific for epitopes from these 2 antigens following pDLI[305,306], serial tracking by pentamer analyses could identify expansions of these leukaemia-specific T-cells in patients at sequential time-points during treatment. Formal analysis of TCRβ repertoire diversity in a large cohort of patients at defined time-points post-HSCT in patients that do, or do not receive pDLI and in comparison with results from a set of age-matched healthy volunteers would begin to address the question of whether there is restricted repertoire diversity following TCD RIC HSCT for AML/MDS, how long restriction persists and whether any changes arise following T-cell add-back.

The above studies of lymphocyte subset composition and function, along with studies of leukaemia-specific T-cell expansion and TCRβ repertoire analysis, could begin to identify why some but not all patients respond to pDLI. In Chapter 3, I observed that one third of patients did not show a sustained improvement in donor T-cell percentage following pDLI and ultimately relapsed. No factors were

identified that associated with chimerism response. Although not discussed in the publication, there were no observed differences in the absolute lymphocyte numbers immediately prior to pDLI in these 2 groups (mean 1363 lymphocytes/μl [1038 – 1689 lymphocytes/μl, CI 95%] in responders and mean 1206 lymphocytes/μl [696 – 1717 lymphocytes/μl, CI 95%] in non-responders, p=0.6). This is relevant as it suggests profound lymphopenia prior to receipt of DLI in responders, which could drive homeostatic expansion of infused donor T-cells, was not the reason for improved chimerism or protection from disease in responding patients. Alternative explanations afforded by the investigations above may enable prediction of responsiveness to pDLI.

The prospective study described above would also analyse the same parameters in patients prior to and following AML relapse. HSCT recipients, who have not relapsed or received DLI and are matched in terms of the time that has elapsed post-HSCT to the relapse cases, as well as age-matched healthy volunteers, could serve as controls. Large-scale studies could identify any immune signatures predictive of relapse and of responsiveness to tDLI. Furthermore, an in depth understanding of the immune deficits that may ultimately allow leukaemic escape from surveillance and how these are corrected in those who respond to tDLI would also be useful in the context of the AML Cell Vaccine. An unanswered question arising from the preliminary data of vaccination in patients receiving tDLI post-HSCT is whether they have "sufficient" immune competence to respond to ACV. A clear definition of immune sufficiency is lacking in this setting but could be addressed by the ability to mount NK and T-cell responses following stimulation with CD80/IL-2 modified blasts, given the previously reported *in vitro* data supporting these lymphocyte populations in mediating leukaemia-specific cytotoxicity[120,121]. Studies using T and NK-cells isolated from patients following tDLI did not however form part of these earlier reports. Analysis of leukaemia-specific cytotoxicity against primary AML blasts *in vitro* following co-culture of T- and NK-cells isolated from patients pre- and post-tDLI with CD80/IL-2 modified AML blasts could provide evidence of immune competence to respond to vaccination and allow an assessment as to whether this is improved by successive tDLI.

As outlined in the discussion in Chapter 5, functional assays are needed to complete the immunological assays in patients from the RFUSIN2-AML1 study. These include measurement of leukaemia-specific cytotoxicity of CFSE-labelled autologous AML blasts by PBMCs from trial subjects. To determine specificity of the response, PHA-activated T cells or EBV-transformed B-cells established from patients could be used as autologous controls. Leukaemia cytotoxicity pre- and post-DLI with/without ACV would be compared. Following similar cultures, ELISpot assays would allow assessment of IFNγ and Granzyme B production. The TCRβ repertoire analyses in both UPNs 14 and 22 require completion for the remaining time points on study follow-up to continue to track clonotypes over time. This analysis also needs to be extended to the other trial subjects. Importantly for UPN 22, TCRβ repertoire analysis using the DTH biopsy site samples could suggest whether any clones that dramatically increase in the peripheral blood are also highly represented in the biopsy site. This could suggest expansion of leukaemia-specific clones that are attracted to the vaccination site at the time of the DTH reaction. These studies will also be more informative following the protocol amendment that permits control vaccinations to be performed in patients, since it would be hypothesised that any clonotypes showing high frequencies in the DTH site would not be prominent in the control injection sites. Finally, another approach to assessing the safety of the ACV will be to consider other patient groups for vaccination. I have written and submitted a clinical trial application for a Phase I study using the ACV to maintain remissions following chemotherapy in AML patients who are not candidates for transplant. This has been approved by the MHRA and relevant ethical bodies and will begin recruitment at King's College Hospital in the near future.

With respect to the findings presented in Chapter 4, the most important issue to address in WT1 vaccinations using CASAC will be to identify methods to increase the consistency and magnitude of immune response induction. This would then permit vaccine efficacy to be evaluated in a tumour model by challenging immunised mice with a murine syngeneic WT1+ AML cell line. Around 2 in 5 (40%) non-tumour bearing mice showed expansion of WT1-specific T-cells following vaccinations with WT1 and CASAC within each experiment and the functional responses observed, such as lysis of WT1-RMF loaded targets, varied in magnitude between experiments. In some of the vaccination studies, no mice responded to vaccination with WT1 peptides and CASAC. Future studies should investigate this inconsistent immune response induction. Optimisation of the immune readouts from these experiments is one factor that

requires consideration. For example, a more sensitive test than pentamer analyses, such as *in vitro* analysis of WT1-specific IFNγ production by PBMCs isolated following vaccination rounds using an ELISpot assay, might yield more information. Secondly, it would be of interest to determine whether there are any signs of an exhausted T-cell phenotype in non-responders to vaccination in contrast with those mice that show expansion of WT1-RMF or OVA-SIINF specific T-cell responses. Individual mice would be ear-tagged to follow their responses. T-cells isolated from peripheral blood and draining lymph nodes after repeated vaccination could be assessed for expression of inhibitory markers (often reflective of T-cell exhaustion), such as CTLA-4, PD-1 and LAG-3[347,348]. An exhausted T-cell phenotype might suggest a requirement for further optimisation of dosing and timing of vaccine administration. Furthermore, it would be relevant to determine whether there is an expansion of the Treg population in draining lymph nodes close to the vaccination sites. Although this population was not expanded in the peripheral blood after repeated vaccinations using CASAC, Class I and Class II WT1/OVA peptides, the lymph nodes may show contrasting findings. Expansion of Tregs within lymph nodes could provide a target for inhibition that may enable greater induction of immune responses (see below).

The dissection of differences in the immune phenotype of T-cells in responsive and non-responsive mice relies of course on the ability to induce WT1-specific responses. The failure to induce WT1-specific responses in some experiments and the inconsistent responses in others suggest that manoeuvres to boost induction of response to vaccination are required. Approaches to consider include (1) addition of other stimulatory signals that may synergise with CASAC components and (2) dampening down or elimination of regulatory signals that may limit response induction. Ways to achieve this are described below. Although principally discussed in the context of maximising induction of WT1-specific T-cell responses using CASAC, these strategies could equally be relevant to enhance leukaemia-specific immune response induction by other types of vaccination, such as the AML Cell Vaccine.

Additional stimulatory signals within the adjuvant cocktail may increase the vaccination efficacy. In the original studies by Wells et al describing CASAC, an agonist anti-4-1BB (CD137) antibody was delivered 1 week after the second vaccination using OVA-SIINF and CASAC to reduce the contraction phase of the

CD8+ T-cell response. Although the size of the memory pool itself was unaffected by the inclusion of the agonist 4-1BB antibody, there was evidence of a more vigorous recall response upon re-challenge with OVA-SIINF in mice that received the agonist antibody[238]. Other members of the Tumour Necrosis Factor Receptor (TNFR) family, such as OX40[349] and GITR[350] have not yet been studied in the context of CASAC for priming and boosting of antigen specific responses. Ligation of GITR can augment CD28-mediated costimulation of T-cells during priming whereas the effect of 4-1BB ligation on T-cell function appears to be greater on antigen-experienced effector T-cells[350]. The apparent non-redundancy of these various costimulatory receptor and ligand functions suggests that inclusion of agonists within CASAC and administration at appropriate time-points (during priming and/or boosting) could have an additive effect to increase the rate of induction and avidity of LAA-specific T-cell responses.

Alternatively, additional TLR agonists could be included in the vaccine. The use of 3 TLR agonists (ligands for TLRs 2, 3 and 9) in the adjuvant cocktail has been explored in the context of vaccination against HIV envelope protein in a mouse model and appeared to improve the quality (functional avidity of the response enabling viral clearance) rather than the magnitude of the response[351]. The inclusion of an additional TLR agonist that binds other receptors to synergise through alternative signalling pathways to activate transcription is also attractive. TLR5 has garnered particular enthusiasm as a target, as its only known ligand is an exogenously-derived microbial component (flagellin, derived from the flagellae of microbes such as *salmonella spp*). Vaccination of mice with tumour cells genetically modified to express flagellin permitted rejection of the parental tumour through activation of CD8+ and CD4+ T-cell responses. Flagellin mediated its effects by activating DCs through ligation of TLR5 and members of the Nod-like receptor (NLR) family, NLRC4 and NAIP5 (neuronal apoptosis inhibitory protein 5)[352]. Recently, a modified version of flagellin administered intravenously in a murine model of lymphoma permitted rejection of established tumour[353]. Combination of this product within the CASAC cocktail could synergise with other TLR signalling pathways to increase efficacy.

A further consideration is delivery of the vaccine components to DCs[354]. The study using flagellin in a whole cell vaccine demonstrated that only flagellin within the irradiated tumour cell vaccine, as opposed

to recombinant flagellin co-administered with irradiated tumour cells, could effectively prolong survival and elicit an adaptive immune response in mice[352]. An explanation could be requirement for the TLR agonist and LAA(s) to be present within the same phagosome to signal through receptors within that particular phagosome. This results in loading of selected peptides from the antigen of interest onto Class II molecules for cross-presentation[355]. This process, termed "associative recognition" of the antigen with PAMPs, suggests that physical linking of the LAA to a TLR may be important to effectively prime T-cell responses[352,355,356]. Covalent linking of TLR agonists such as CpG ODN to immunising peptides has been shown to improve immunogenicity[357]. This may be more straightforward to achieve in the context of immunisation using a single epitope but more complex if a multi-epitope vaccine such as the overlapping WT1 peptide pool is used for vaccination. Alternatively, delivery of peptides and adjuvant components within liposomes may avoid the need for conjugation but still enhance delivery of antigen and PAMPs to the same endosomal compartment[358]. More recent advances in vaccine design have explored the potential for nanoparticles/microparticles engineered to contain antigen and adjuvants, essentially mimicking microbial structures and effectively triggering DC activation following uptake, with resultant cross-presentation to T-cells[359].

The strategies described above suggest ways to provide additional positive signals to drive immune response induction with CASAC. Strategies to reduce or eliminate negative, regulatory influences limiting immune response induction should also be studied in combination with CASAC. This may prove to be the most effective means to augment induction of T-cell responses against a self-antigen such as WT1, given that tolerance mechanisms will be present to control WT1-specific T-cell reactivity. As discussed above, future studies should include investigation of the absolute numbers and frequencies of Tregs relative to effector T-cells in the draining lymph nodes of vaccination sites. Subsequent *in vitro* culture of lymph node-derived cells with immunising WT1 peptides could identify antigen specific proliferation of Tregs. Co-culture of these Tregs with effector T-cells would determine if they are capable of suppressing proliferation of WT1-specific T-cell responses. Not only could repeated exposure to WT1 Class II peptides stimulate Treg expansion, there is also evidence that activation of DCs, for example by TLR agonists, can result in their increased expression of indoleamine oxygenase (IDO)[360]. Exposure to IFNα and IFNγ can also result in increased expression of IDO by DCs that acquire immunoregulatory function, suppressing

effector T-cells and inducing/activating Tregs[361]. One way to determine if this is the case following repeated CASAC vaccination could be to quantify IDO expression in DCs within the draining lymph nodes in vaccinated recipients relative to controls (and responders versus non-responders)[362]. In combination, increased IDO transcripts and Treg frequencies would provide a rationale for IDO inhibition and/or Treg depletion. Administration of 1-methyl tryptophan (1-MT) is being explored as a cancer therapy due to its ability to inhibit IDO[361,363]. Co-administration of 1-MT along with vaccination might therefore increase the efficacy of CASAC vaccinations targeting WT1.

An alternative to IDO inhibition is depletion of Tregs. Administration of an anti-CD25 antibody prior to therapeutic vaccination with tumour peptide-loaded DCs in a murine leukaemia model was associated with greater survival, compared to that observed in mice immunised without anti-CD25 antibody pre-treatment[364]. The use of CD25-depleting antibodies or low dose cyclophosphamide to diminish regulatory T-cell activity may be a further means to boost vaccination responses. WT1-specific Tregs have been observed in the peripheral blood of patients with AML and have been expanded *in vitro* by culturing healthy donor PBMCs with WT1 peptides[365]. Therefore in the context of CASAC and WT1 vaccinations as immunotherapy for AML, not only would avoidance of inducing WT1-specific Tregs by repeated vaccination be relevant, elimination of existing WT1-specific Tregs may also be necessary. Indeed, higher frequencies of total peripheral blood Tregs have been reported in AML patients relative to controls[366], suggesting that Treg depletion is particularly relevant for immunotherapy of AML patients[367-369]. The finding that low dose cyclophosphamide therapy in a murine breast cancer model facilitated expansion of high avidity T-cells specific for human epidermal growth factor receptor 2 (*her2),* through depletion of Tregs, is particularly tantalising[57]. Immunisation could combine with Treg depletion to expand high avidity tumour-reactive T-cells.

Lastly, blocking antibodies to immune regulatory receptors such as CTLA-4[109] and PD-1[370] (or its ligand PDL-1[371]) have already shown promise in clinical trials of immunotherapy for a variety of malignancies. Indeed dual blockade of CTLA-4 and PD-1 has recently been evaluated in a Phase I clinical trial of melanoma, showing objective responses in over 40% of patients and an acceptable side effect profile[372].

Whether cancer vaccinations may synergise with these antibodies to promote priming and amplification of tumour-specific T-cell responses is an approach that is being explored in clinical trials[373]. In the context of AML, murine models have already implicated PD-1/PDL-1 interactions in the impaired immune response to leukaemia[374,375]. Therefore combining CASAC vaccinations against WT1 with exposure to anti-CTLA-4 and/or anti-PD1 antibodies may promote induction of WT1-specific T-cell responses. This should be considered upon translation to the setting of therapeutic vaccination in a tumour model. The optimal timing and sequence of exposure to the vaccine and anti-CTLA-4/anti-PD1 antibodies would need full investigation in order to allow these approaches to synergise effectively.

When the rate and magnitude of immune response induction in non-tumour bearing mice has been improved, efficacy of CASAC and WT1 vaccinations should be assessed in a murine model of AML. The C57BL/6 derived WT1+ AML cell line FBL-3 could serve as a suitable pre-clinical model to assess both prophylactic and therapeutic vaccinations before considering translation into early phase clinical trials.

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Appendices